INVESTIGATIONS OF CEA ON HUMAN COLON CARCINOMA CELLS

INVESTIGATIONS OF THE EXPRESSION OF CARCINOEMBRYONIC ANTIGEN AT THE SURFACE OF CULTURED HUMAN COLON CARCINOMA CELLS

By

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ABSTRACT

A number of cellular products present during fetal development, but absent from normal adult tissues, have been shown to be re-expressed in cancer cells. One example of these oncofetal substances is carcinoembryonic antigen (CEA). Studies were undertaken to examine the expression of CEA at the surface of human colon carcinoma cells grown *in vitro* and to develop a radioimmunoassay for quantitation of CEA and antibodies to CEA in the serum of cancer patients.

Antibodies specific for CEA were prepared in goats and these antibodies were found to induce polar redistribution or capping of the antigen. As with other systems in which polar redistribution of surface molecules have been studied, the capping was temperature-dependent and required an intact microfilament system. Fluorescent-labeled antibodies were utilized to demonstrate that while CEA would undergo capping, blood group antigen A did not, hence these antigens exist as separate molecules at the cell surface. The capping process was further characterized using ^{125}I-labeled antibodies and it was demonstrated that upon capping the majority of cell

surface CEA underwent endocytosis. The ability to specifically remove CEA from the cell surface with antibody was used to demonstrate a rapid reappearance of CEA on the tumor cell surface, and this reappearance appeared to require protein synthesis.

A precise quantitative radioimmunoassay for CEA was developed and used to determine the amount of CEA expressed on cell surfaces. Various strains of cells were established *in vitro* which differed in the amount of CEA they produced. Two strains which differed in the amount of CEA expressed at their cell surfaces were shown to be equally tumorigenic in nude mice, which suggested a lack of correlation between CEA production and tumorigenicity.

The radioimmunoassay was also used to study the control of genetic expression of CEA. There was a direct correlation between the amount of cell surface CEA and the amount of CEA secreted into the culture medium. Control over the level of CEA expressed by various strains appeared genetically stable. Yet, a number of lines of evidence suggested that the parental population from which the strains were derived was heterogeneous with respect to CEA synthesis.

The effects of various inducing agents on CEA expression by various cell strains was examined. One strain (HCT-8 Nu2), a very low CEA producing strain,

could be induced to express high levels of CEA by inclusion of theophylline in the culture medium. This effect appeared after three days of incubation and reached a maximum after five days. Enhanced expression was dose-dependent and time-dependent, requiring continual presence of the drug. The effect also appeared to require continual protein synthesis and did not cause marked alteration of cell morphology or growth. It was demonstrated that the effect was not density-dependent and did not appear to be due to selective proliferation of a high expressor population. Further, the effect could not be mimicked with dibutyryl cyclic adenosine monophosphate. Similarly, another strain (HCT-8R) could be induced to produce higher levels of CEA with bromodeoxyuridine (BrdU). This effect was not as dramatic as the theophylline effect and only appeared transiently. The response to BrdU was dose-dependent.

The specific inhibition of binding of ^{125}I-labeled anti-CEA antibodies by unlabeled anti-CEA antibodies was used to demonstrate that no antibodies to CEA could be detected in control or cancer patient sera. The radioimmunoassay was also examined to determine its ability to quantitate the amount of CEA in serum from cancer patients and controls. It was determined that this test could measure comparable ranges of standard reference CEA, obtained from international or marketed

sources. The results obtained from tests of patient sera closely correlated with results obtained using a marketed assay kit. A limited number of sera from patients was examined for CEA using the assay. Comparable percentages of patients with CEA-related cancers were found positive by my assay as reported in studies using standard assays. However, my assay appeared to have greater specificity than standard assays in that a lesser percent of patients without CEA-related cancers were positive.

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I dedicate this thesis to my parents,
especially my mother,
who knows cancer more intimately than I.

LITERATURE REVIEW

I. Historical Background

The search for molecular markers of the neoplastic cell has led to the recognition of a number of tumor-associated antigens, including oncofetal antigens (Rev. in Alexander, 1972; Coggin & Anderson, 1974; Constanza & Nathanson, 1974; Lausch & Rapp, 1974). Oncofetal antigens may be defined (Constanza & Nathanson, 1974) as molecules, usually identified by immunological means, that are found in both malignant and embryonic or fetal tissue but are absent or undetectable in nonmalignant tissues.

The presence of embryonic antigens in tumor cells was first described in 1906 when G. Schone (1906) reported that mice which had been injected with fetal tissues rejected transplants of tumor tissue which otherwise grew and killed. Adult tissues did not evoke this response. However, outbred mice were used in these studies and the results cannot be attributed solely to fetal antigens, since tissue and histocompatibility antigens were also present. In the 1930's, humoral antibodies that cross-reacted with fetal and tumor tissues were identified

(Hirszfeld *et al.*, 1929, Witebsky, 1930). More recently, using inbred strains of mice and rats, several groups (Alexander, 1972; Baldwin *et al.*, 1974; Coggin *et al.*, 1971; Hellstrom and Hellstrom, 1975; Steele and Sjogren, 1974) have clearly identified tumor-associated embryonic transplantation antigens.

A number of human oncofetal antigens have been identified. The two which have been studied in detail are alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA).

In 1963, Abelev *et al.* (1963) discovered that one of the abnormal serum proteins synthesized by a chemically-induced hepatoma in mice was antigenically identical to an α-globulin present in embryonic and neonatal mouse serum but absent from the adult mouse. Within one year, similar material had been found by Tatarinov (1964) in the serum of patients with primary hepatoma. Literature concerning AFP and oncogenesis has been recently reviewed (Abelev, 1971, 1974; Masseyeff, 1972; Ruoslahti *et al.*, 1974; Uriel, 1969; Wepsic & Sell, 1974; Sell *et al.*, 1976) and hence will not be dealt with here.

Rather, the primary purpose of this section is to review literature concerning CEA. In 1965, Phil Gold and Samuel Freedman (1965a) demonstrated the presence of tumor-specific antigens in extracts of human colon carcinoma which were not present in normal colonic tissues. Subsequently, they demonstrated the presence of identical antigens in

all malignant tumors arising from entodermally derived epithelium of the gastrointestinal tract (Gold & Freedman, 1965b). But these antigens were absent from other adult tissues (Gold & Freedman, 1965b). Further, experiments with fetal tissues (Gold & Freedman, 1965b) indicated that identical antigens were also found in fetal gut, liver and pancreas between the second and sixth month of gestation. Since these components were absent from normal adult gastrointestinal tissues but found in both fetal and cancerous gastrointestinal tissues they were named "carcinoembryonic" antigens (Gold & Freedman, 1965b).

With the development of radioimmunoassay (RIA) techniques, it was shown (Thomson *et al.*, 1969) that CEA could be detected in the serum of patients with gastrointestinal malignancies but not in the serum of normal individuals or patients with other cancers. The possibility that CEA assays might provide a diagnostic test for colo-rectal cancer provided impetus for extensive clinical trials and also for investigations concerning the physical, chemical and immunochemical characteristics of CEA.

II. Physiochemical Characterization of CEA

CEA, derived from human metastatic colorectal carcinomas, is a glycoprotein soluble in perchloric acid

and strong salt solutions (Krupey *et al.*, 1968). It has been typically extracted and isolated from homogenized tumor tissue by solubilization with perchloric acid followed by gel filtration (Coligan *et al.*, 1972; Terry *et al.*, 1974). An independent approach to CEA isolation has been taken by Rosai *et al.* (1972). They first prepared a crude membrane fraction from colon tumors, solubilized the CEA with lithium diiodisalicylate, and removed the proteins by phenol extraction. They then subjected the mixture to chromatography and electrofocusing to yield purified CEA. Other investigators have utilized Concanavilin A sepharose affinity chromatography to purify CEA (Harvey & Chu, 1975). Each step of the purification procedure employed monitoring for CEA using pre-existing antiserum to CEA. Thus, as will be discussed in the section on CEA's immunochemistry, CEA is antigenically defined.

CEA has a sedimentation constant of 7-8S and a molecular weight of about 200,000 as judged by gel chromatography (Coligan *et al.*, 1972; Krupey *et al.*, 1972; Pusztaszeri & Mach, 1973). Its electrophoretic mobility at pH 7-8.5 is that of a plasma β-globulin (Krupey *et al.*, 1968). Isoelectric focusing of CEA preparations reveal much heterogeneity in electrical charge (Coligan *et al.*, 1973; Turberville *et al.*, 1973; Rev. in Rogers, 1976). Recently an isomeric species of CEA was isolated

and characterized (Plow & Edgington, 1975). This molecule, called CEA-S, was shown to be homogeneous with respect to size, density and charge (Plow & Edgington, 1975).

The amino acid composition of CEA represents that of a typical glycoprotein, with a high content of aspartic acid, glutamic acid, threonine and serine (Krupey *et al.*, 1968; Terry *et al.*, 1974). The N-terminal sequence of CEA has been determined by a number of groups (Terry *et al.*, 1972, 1974; Holyoke *et al.*, 1975) and there is agreement on the first 30 amino acid residues.

Unsuccessful attempts were made to split CEA into separate protein chains by urea, 6 M guanidine HCl and detergent treatment alone or after reduction of disulfide bonds (Hammarström *et al.*, 1975). This evidence coupled with the consistent results of amino terminal sequencing suggested that CEA probably consists of a single polypeptide chain of about 800 amino acids (Neville & Laurence, 1974).

Carbohydrate analysis of CEA has been reported (Terry *et al.*, 1974; Neville & Laurence, 1974; Coligan *et al.*, 1978; Egan *et al.*, 1976) and it has been determined that CEA is composed of 45-57% carbohydrate by weight. The molecule is believed to demonstrate much branching. Further, the sialic acid content varies from sample to sample (Krupey *et al.*, 1968). Other sugars also demonstrate variation and this may reflect differences between tumors

due to breakdown or synthetic processes *in vivo* or differences in the isolation or analytical procedures employed (Terry *et al.*, 1974; Neville & Laurence, 1974).

There are two common ways by which carbohydrates link to peptides in mammalian glycoproteins (Spiro, 1970). Since the attachment of the sugar to the peptide chain of CEA was found to be resistant to mild alkali treatment (Terry *et al.*, 1974; Neville & Laurence, 1974) it is believed that the carbohydrate-peptide bond occurs through an asparagine-N-acetylglucosamine linkage.

III. Cellular Location of CEA

The cellular location of tumor-associated antigens is important in terms of the potential host response to them and their possible function. The cellular location of CEA was determined by immunofluorescent studies of unfixed frozen sections of tumor tissue treated with fluorescein-conjugated rabbit anti-CEA sera (Gold *et al.*, 1968). Fluorescence was sharply localized to the plasma membrane of the tumor cells. Similar observations were made by von Kleist & Burtin (1969), but the majority of fluorescence appeared limited to the free membranes of cells bordering the lumina of the glands. In 1970, Gold *et al.* (1970) treated tumor cells with ferritin-labelled antibody to CEA and used ultrastructural analysis

to localize CEA to the glycocalyx, or "fuzzy-coat" of the tumor cell membrane. The possibility existed, though, that these studies were only detecting CEA which had been secreted and absorbed onto the cell surface.

This possibility was eliminated when CEA was demonstrated on the surface of colon carcinoma cells which had been grown and maintained *in vitro* (Herberman *et al.*, 1975).

Our current understanding of the structure of cell membranes has been dramatically advanced by the fluid mosaic model proposed in 1972 by S.J. Singer and G.L. Nicolson. Based on thermodynamic considerations of macromolecular systems and in light of the then available experimental evidence, they concluded that membranes were composed of a mosaic structure of heterogeneous globular amphipathic proteins partially or totally embedded in a matrix consisting of a fluid lipid bilayer. An important implication of this model was that molecules of the membrane would be capable of lateral movement within the plane of the membrane. Hence, the membrane must be considered a very dynamic structure. Much evidence has accumulated to support the concept that molecules are capable of lateral movement in the plane of the membrane. The majority of studies demonstrating redistribution of surface molecules have utilized antigenic determinants on lymphocytes (Frye and Edidin, 1970; Taylor *et al.*, 1971;

Loor *et al.*, 1972; Kourilsky *et al.*, 1972); however, recently certain antigens of other types of cells were found to be redistributed by antibody binding (Joseph & Oldstone, 1974; Comoglio & Guglielmone, 1972).

IV. Immunochemistry of CEA

Carcinoembryonic antigen has been defined immunologically. Glycoproteins are extracted, isolated and purified from a tumor source, used to immunize any of a variety of animal species and the resulting antiserum is shown to precipitate and produce a line of identity between the immunizing material as well as a reference preparation of CEA. Thus CEA is circularly defined. Most studies have employed demonstrations of identity to CEA obtained from Gold and coworkers but recently an international standard of CEA has been made available (Laurence *et al.*, 1975).

Initially CEA was believed to consist of a single homogeneous glycoprotein (Krupey *et al.*, 1968). But subsequent studies have demonstrated that preparations of CEA exhibit heterogeneity (Rev. in Rogers, 1976). In addition to the already mentioned physiochemical heterogeneity observed, with respect to size, charge, density and isoelectric point, differences have been described between antigenic preparations derived from adenocarcinoma of the colon and preparations derived from fetal tissue

(Rule & Goleski-Reilly, 1974). Further heterogeneity (discussed in the next section) has been observed with respect to the presence of blood-group antigens (Holburn *et al.*, 1974).

Antisera prepared against CEA is usually absorbed to render it CEA-specific. This technique has its limitations and consequently the antiserum may contain residual antibodies or antibodies that react with other non-CEA contaminants (Rogers, 1976). A number of normal tissue components which cross-react with CEA have consequently been identified and have been called NGP (Mach & Pusztaszuri, 1972), NCA (von Kleist *et al.*, 1972), FSA (Hakkinen, 1972), CCEA-2 (Darcy *et al.*, 1973), BCGP (Kuo *et al.*, 1973) and NCA-2 (Burtin *et al.*, 1973). Recent results suggest that NGP, NCA and CCEA-2 may be identical (Edgington *et al.*, 1976). Hence variations in antisera contribute to the observed heterogeneity of CEA.

Ultimately, the heterogeneity of CEA is believed to be a major contributing factor to the lack of specificity which has severely limited the diagnostic value of the CEA radioimmunoassay (Rogers, 1976, and discussed in a later section). For this reason, the major research thrust concerning CEA has focused on the immunochemistry of the molecule, with primary emphasis on elucidating which antigenic preparation or antigenic determinant of CEA is the most cancer-specific. Studies of this nature

are exemplified by work on the homogeneous isomeric species of CEA called CEA-S (Plow & Edgington, 1975; Edgington *et al.*, 1975, 1976a, 1976b). Whether CEA-S represents a more colon cancer-specific form of CEA, as claimed by its investigators (Edgington *et al.*, 1975) or not (Zamcheck *et al.*, 1975) is currently an undecided issue.

Removal of sialic acid from CEA by neuraminidase treatment, while removing much of the charge heterogeneity (Coligan *et al.*, 1973), did not alter the radioimmunoassayable activity per unit of mass of the material (Coligan *et al.*, 1973; Hammerstrom *et al.*, 1975; Banjo *et al.*, 1974). Fucose was removed by controlled periodate treatment and immunological reactivity was unimpaired (Coligan *et al.*, 1973). Consequently, neither fucose nor sialic acid appear to be essential for specific activity. Recent studies have shown that 85% of the carbohydrate residues in CEA can be removed without appreciable loss of antigenic activity (Hammerstrom *et al.*, 1975; Coligan & Todd, 1975), showing that the immunodeterminant is present in the innermost carbohydrate residues or in the protein sub-structure (Rogers, 1976). In support of this idea were recent studies demonstrating that the integrity of the protein portion of CEA was important for high immunological activity (Westwood *et al.*, 1974; Westwood & Thomas, 1975). Thus the activity was abolished by treatment of CEA with 0.5 M NaOH at 20°C and reduced to 3-5% of the activity of untreated

CEA on cleavage of the disulphide bonds.

Recently, using quantitative immunochemical analysis employing absorption and competitive inhibition radioimmunoassays, Leung *et al.* (1977) delineated three classes of CEA antigenic determinants. The first class consists of determinants found on all preparations of CEA and these were called group-specific determinants. The second class was called cryptic determinants because antibodies to them could be absorbed by SDS-unfolded soluble CEA or a membrane-associated species of CEA (Rosai *et al.*, 1972) but not by conventional soluble forms of CEA. The last class of determinants was called species-specific and were detectable only on the membrane-associated CEA. The presence of unique antigenic determinants on membrane-associated CEA has been ascribed to its conformation (Leung *et al.*, 1977) but whether or not these determinants will provide a more colon cancer-specific antigen has not been determined.

V. Relationship Between CEA and Blood Group Antigens

Purified preparations of CEA were reported to contain antigenic determinants in common with blood group A (Gold *et al.*, 1972; Turner *et al.*, 1972; Gold & Gold, 1973). This conclusion was based on the observations that IgM anti-A antibodies bound to CEA when assayed by

radioimmunoelectrophoresis. The findings were supported by immunochemical studies which suggested that CEA molecules were incomplete blood group substances of the ABO system (Simmons & Perlman, 1973). Holburn *et al.* (1974) also detected blood group antigens A, B, Le^a or Le^b in purified preparations of ^{125}I-labeled CEA in reactions utilizing blood group antibodies. In all cases, the blood group antigens detected were consistent with the known blood group of the patients. These authors concluded that the determinants of the blood group antigens and of CEA share the same molecule (Holburn *et al.*, 1974).

Alternatively, it was possible that the blood group antigens were extracted and purified along with CEA as non-covalently attached molecules as suggested by Terry *et al.* (1974). This was supported by recent studies which demonstrated that CEA and blood group antigens have similar isoelectric focusing patterns but the substances were separable by absorption with affinity gels (Cooper *et al.*, 1974). Denk *et al.* (1974), employing immunofluorescence on fixed tumor tissues, demonstrated that when present on the same cell, they were in different locations. While CEA was demonstrated only on the lumen-oriented surface of the cell, the blood group antigens were distributed over the entire cell surface.

Thus the relationship between CEA and blood group antigens remains to be determined.

VI: Factors Affecting the Genetic Expression of CEA

Cancer has often been viewed as a problem of anomalous differentiation (rev. in Markert, 1968; Pierce, 1970; Coggin & Anderson, 1974; Anderson & Coggin, 1974; Braun, 1975). Indeed, the degree to which patterns and evidence of differentiation are lost in malignant cells is part of the basis for description and identification by pathologists. Further, numerous analogies between the properties and behavior of cancerous and embryonic cells exist (Rev. in Uriel, 1975). The discovery of the reappearance of fetal products, such as AFP and CEA, in adult neoplasms has renewed interest in these ideas.

The early work of Gold & Freedman (1965b) led these investigators to hypothesize a mechanism of derepressive-dedifferentiation of normal fetal genes in malignant tumors of the entodermally derived epithelium of the gastrointestinal tract. The derepressive mechanism was based on the model proposed by Jacob & Monod (1961).

Very little information concerning the genetics of CEA expression is available, and most of the work in this area has been confined to theorizing.

A series of studies conducted by Rule and coworkers (Rule & Goleski-Reilly, 1973, 1974; Rule & Kirch, 1976) employing comparisons of "fingerprints" obtained by CEA radioimmunoassay and isoelectric focusing patterns of

saline extracts of fetal gut tissue and colon tumors. I suggested that more than one CEA gene exists. And further, since individual colon tumors exhibited random qualitative and quantitative differences in gene product patterns, they suggested that colon cancer involved the loss of integrator and controller genes which would regulate the spectrum and quantity of molecules with CEA determinants (Rule & Kirch, 1976).

These concepts, while interesting, lack conclusive proof. As mentioned, much of the heterogeneity of CEA, viewed by Rule as distinct gene products (Rule & Kirch, 1976), has been attributed to variation in the carbohydrate moieties (Rogers, 1976). The addition of carbohydrate to glycoprotein is believed to occur as a post-ribosomal event and, therefore, is only partly under genetic control (Spiro, 1970). Further, Rule points out (Rule & Kirch, 1976) that her studies employ examination of final gene products which may have undergone extensive modification post-transcriptionally and post-translationally (Davidson & Britten, 1974).

One approach toward elucidation of cellular controls affecting gene expression has involved the use of various chemicals to enhance gene product synthesis. These "induction" studies are exemplified by work conducted on Friend virus-induced erythroleukemic cells. It was noted that these cells when incubated in medium containing

dimethyl sulfoxide (DMSO) would mature into hemoglobin synthesizing normoblasts (Friend *et al.*, 1971). DMSO, the "inducing-agent", caused these cells to differentiate and this process was accompanied by increased synthesis of hemoglobin and a decrease in malignancy (Friend *et al.*, 1971). Recent work in this area has led to the precise characterization of the differentiation program induced in these cells by DMSO (Gusella *et al.*, 1976).

Besides the inductive effects of cryoprotective agents on erythroid differentiation, the effects of halogenated pyrimidines on differentiation and malignancy have been examined. Silagi & Bruce (1970) noted that incubation of mouse melanoma cells in medium containing 5-bromodeoxyuridine (BrdU) would cause a loss of pigment producing ability and a concomitant loss of tumorigenicity.

For many years it had been recognized that murine leukemia virus (MLV) could be activated by certain carcinogenic stimuli, aging or spontaneously. Virus negative lines, established from high leukemic AKR mice, were shown to rarely produce infectious MLV spontaneously (Lowy *et al.*, 1971). The frequency of spontaneous activation was estimated to be 10^{-8} to 10^{-9}. Lowy *et al.* (1971) showed the activation rate could be increased by about 10^{6} times by exposing growing cultures to 20-100 µg of IrdU or BrdU/ml for 24-48 hours. This induction provided strong evidence that the genetic information for C-type virus

production was present in most mouse cells and that this information could be transmitted vertically for many generations without being expressed.

These studies led Aaronson *et al.* (1971) to demonstration that clones established from Balb/c mouse embryo cells could each be induced to produce C-type particles. Hence, it appeared that all mouse cells carried a complete viral genome.

Studies concerned with the etiology of mammary tumorigenesis have relied largely on the mouse model system in which murine mammary tumor virus (MMTV) has been found to be an important factor. Studies carried out by Parks & Scolnick (1973) indicated that while clones of cells established from a spontaneous mouse mammary tumor contained comparable levels of MMTV DNA they expressed markedly different levels of MMTV RNA and virion protein. These results suggested that MMTV expression might be regulated by cellular controls acting at the level of transcription.

Since hormones were also known to play a major role in mammary tumorigenesis (Bern & Nandi, 1961) an attempt was made to stimulate MMTV production in these cells with steroids. It was found that dexamethasone stimulated production of MMTV 10 to 100 fold and appeared to induce a viral reverse transcriptase (Parks *et al.*, 1974). Recent work (Scolnick *et al.*, 1976) has indicated that increased viral production may

be attributed to an increased rate of synthesis of MMTV RNA.

Since many hormones and mediators of change in genetic expression may act through second messengers, such as cyclic AMP, these substances and their inhibitors have recently received attention as potential inducing agents. It was recently demonstrated that dibutyryl cyclic AMP (dbcAMP) would stimulate the synthesis of melanin in a number of cell lines (Johnson & Pastan, 1972; Helson *et al.*, 1974; Takeuchi & Kajishima, 1976).

In light of the success obtained using induction techniques in elucidating controls of genetic expression, it would be interesting to establish an inductive system for CEA.

VII. Antigenicity of CEA in the Human Host

CEA's natural antigenicity in the human is far from clear. Originally, Gold (1967) reported the presence of circulating antibodies to CEA in patients with non-metastatic colon carcinoma and in pregnant women. Other studies failed to confirm the presence of specific human antibody to CEA (Collatz *et al.*, 1971; LoGerfo *et al.*, 1972). It has been pointed out that Gold's antigenic material was a crude perchloric extract of colonic carcinomas and that when purified CEA has been used, no

specific CEA antibody was detected (Collatz *et al.*, 1971; LoGerfo *et al.*, 1972).

Subsequently, Gold reported the presence of human anti-CEA antibodies using radioimmunoelectrophoresis (Gold *et al.*, 1972). He noted that patients with circulating anti-blood group A antibodies often gave falsely positive results, and much of the anti-CEA reactivity could be removed by absorption of sera with group A erythrocytes. However, even after absorption, several patients still demonstrated anti-CEA activity.

More recently, MacSween (1975) demonstrated the binding of globulins to ^{125}I-CEA in sera obtained from normal and cancer patients. Binding appeared to be weak and of low affinity and appeared to be predominantly IgM. Hence, this antibody activity may only represent cross-reactivity by antibodies directed against similar antigens (MacSween, 1975).

Cell-mediated immunity to CEA has also been investigated. Hollinshead *et al.* (1970) reported the occurrence of delayed hypersensitivity to crude perchloric extracts of colon carcinomas but not to purified CEA. A recent study (Straus *et al.*, 1975) demonstrated that purified CEA did not significantly inhibit the migration of leukocytes from colon cancer patients and patients with Crohn's disease or ulcerative colitis.

Hence, the question of CEA's antigenicity is unresolved and the possibility still exists that humans are tolerant to this oncofetal substance.

VIII. Clinical Significance of CEA

Ever since the initial demonstration reported by Thomson *et al.* (1969), that the serum of 97% of patients with cancer of the colon contained elevated levels of CEA, attempts have been made to investigate the usefulness of assays for CEA in the detection of malignant disease. The major potential uses for CEA assays are: 1) screening of the "normal" population for the presence of undetected cancer; 2) aiding in the differential diagnosis of patients with suspected cancer; 3) following of patients immediately after surgery or other therapy to determine the therapy's effectiveness, and 4) to follow patients for a prolonged time after apparent curative therapy to detect recurrent disease (Terry *et al.*, 1974).

A number of different assays are presently being used to measure the concentration of CEA in plasma or serum. Most are radioimmunoassays (RIA) which utilize the general principle of inhibition of binding of a known amount of radiolabeled purified CEA (Egan *et al.*, 1974). Antibody and bound CEA have been precipitated either chemically (Thomson *et al.*, 1969; Hansen *et al.*, 1971) or

with anti-immunoglobulin (Egan *et al.*, 1972). A modification developed by LoGerfo *et al.* (1971) uses zirconyl phosphate gel (Z-gel) to separate antigen-antibody complexes.

The original optimism about the specific occurrence of CEA in entodermally derived tumors of the digestive tract (Gold & Freedman, 1965b) and in the serum of patients with such tumors (Thomson *et al.*, 1969) has not been sustained (Rev. in Terry *et al.*, 1974; Fuks *et al.*, 1974; Zamcheck, 1975; Zamcheck, 1976). Although many studies have confirmed the high incidence of CEA positivity (60-90%) associated with gastrointestinal cancers (Moore *et al.*, 1971; LoGerfo *et al.*, 1971; Reynoso *et al.*, 1972; Ohar *et al.*, 1972), a high proportion of patients with other malignancies (LoGerfo *et al.*, 1971; Pusztaseri & Mach, 1973; Moore *et al.*, 1971a; Laurence *et al.*, 1972) and with inflammatory conditions (Rule *et al.*, 1972; Zamcheck *et al.*, 1972), demonstrate above "normal" serum CEA levels. Further, approximately 10% of healthy normal individuals demonstrate elevated levels of CEA (Moore *et al.*, 1971; LoGerfo *et al.*, 1971; Laurence *et al.*, 1972; Costanza *et al.*, 1973).

In support of CEA's non-specificity for colon cancer has been the finding of CEA, indistinguishable from Gold's CEA, in normal tissues (Pusztaseri & Mach, 1973; Martin *et al.*, 1972; Burtin *et al.*, 1972; LoGerfo & Herter, 1972), saliva (Martin & Devant, 1973) stool (Elias *et al.*, 1974) and meconium (Rule, 1973).

These results demonstrating the lack of specificity of CEA assays has precluded their use as a general population screen for colon cancer. Even if the percent of false positive tests could be decreased, the usefulness of this assay as a screen for early cancer is limited by the demonstration that less than 50% of early, highly curable (Dukes A) colonic carcinomas were associated with elevated CEA levels (Zamcheck, 1974; LoGerfo *et al.*, 1972a; Ohar *et al.*, 1972). This result was recently confirmed by investigations jointly conducted by the Canadian and American National Cancer Institutes (Miller, 1974). Thus, CEA levels tend to rise with tumor mass or spread (Mach *et al.*, 1973) which make the assay a better test for widespread malignancy.

These results also weaken the argument for the use of CEA tests as a diagnostic procedure. But there is recent evidence to suggest that serial CEA determinations which indicate rising positive levels of CEA may be of diagnostic value (Ohar *et al.*, 1972; Laurence *et al.*, 1972; LoGerfo *et al.*, 1972a).

Holyoke (1975) stated that preoperative CEA levels provided useful prognostic information. They found that only one of twenty patients who had CEA levels less than 2.5 mg/ml developed a recurrence after 18 months. Six of eleven patients with intermediate CEA levels (2.6-7 mg/ml) had tumor reappearance after an average of

11 months, while seven of nine cases with greater than 7 mg of CEA per ml had recurrence on an average of 8.4 months after surgery. So, in general, the higher the CEA level, the poorer the prognosis.

There is general agreement (Neville & Laurence, 1974) that currently one of the most important areas where the monitoring of CEA levels has proven most beneficial is in detecting residual and/or recurrent tumors. Successful surgical removal of tumors was followed by a decline to normal CEA levels in a matter of days (Holyoke *et al.*, 1972; Laurence *et al.*, 1972). Incomplete surgical removal of tumors resulted in some decline in CEA values which, however, never returned to normal (Holyoke *et al.*, 1972). During follow-up of postoperative "cured" patients, rising CEA levels preceded clinical evidence of tumor recurrence by 2 to 18 months (Holyoke, 1974; Mach *et al.*, 1974; Dykes *et al.*, 1974; MacKay *et al.*, 1974; Sugarbaker *et al.*, 1976).

Preliminary studies (Holyoke *et al.*, 1972; Bagshawe *et al.*, 1973; Skarin *et al.*, 1974) also suggest that serial CEA determinations may also prove of prognostic value in monitoring the effects of radio- and chemotherapy.

The nonspecificity demonstrated by the CEA RIA which has limited its effective use for the detection and diagnosis of colon cancer has been attributed to many factors. Among these, where improvement has been sought,

are the reproducibility of the assay, how closely one CEA assay agrees with other assays performed in different laboratories, and the sensitivity and specificity of an assay.

A recent study (Vrba *et al.*, 1975) has shown that the calculated serum CEA concentration could vary by three orders of magnitude depending on the CEA standard, the amount of sera tested and on the particular anti-CEA used in the assay. This study has emphasized the necessity to standarize reagents and has led to the establishment of an international standard of CEA (Neville & Laurence, 1974; Laurence *et al.*, 1975).

Standardization has been employed as one means of circumventing many of the problems, such as lack of tumor specificity, which have also been attributed to the heterogeneity of CEA (Rogers, 1976). This heterogeneity, in turn, has been ascribed to the non-specific manner in which CEA has been defined.

Another approach to this problem has made use of a particular homogeneous species of CEA, CEA-S (Plow & Edgington, 1975), which appeared to be more specific for colonic carcinoma (Edgington *et al.*, 1975).

Similar attempts to determine which antigenic determinant(s) of CEA may be more cancer-specific has resulted in the recent demonstration (Leung *et al.*, 1977) that a membrane-associated form of CEA, CEA-M (Rosai *et*

al., 1972), contains unique antigenic determinants which are not expressed on soluble forms of CEA. The existence of these sites on CEA-M have been attributed to the molecules conformation (Leung *et al.*, 1977) and it would appear that soluble forms of CEA express normally "cryptic" sites which are believed to be exposed upon extraction (Leung *et al.*, 1977).

The specificity for colon cancer demonstrated by the CEA-S assay may not indicate that it indeed represents a more tumor-specific antigenic determinant. It has been argued that similar results may be obtained by using a higher threshold in the regular CEA assay (Zamcheck *et al.*, 1975). Indeed, a marked reduction in the number of "false-positives" results with the CEA assay if 5 rather than 2.5 ng/ml of CEA were used (Zamcheck *et al.*, 1972a). This result would be especially dramatic if "late" colonic cancers were selected (Zamcheck *et al.*, 1975).

Hence, another problem presented by assays of this type involve determining what amount of CEA constitutes the "normal" range. Clearly this will effect the specificity of the assay. The classic statistical technique of defining normal in terms of the mean plus or minus two standard deviations has recently received much criticism (Sackett, 1973, 1975). This approach appeared to lack both statistical and clinical validity, for as the number

of laboratory tests increases, the likelihood that a patient would continue to be classified as normal rapidly diminishes (Murphy, 1972).

Finally, it would appear that the use of CEA as a tumor marker substance will require more detailed studies of its structure combined with studies of its clinical relevance.

IX. Purpose of this Study

In the preceding pages, an attempt has been made to review the literature concerning CEA and to indicate areas where problems remain unresolved. The bulk of CEA research has focused on immunochemistry and the development and clinical application of assays for CEA. At a workshop on CEA, it was generally agreed that little or nothing was known about the biology and function of CEA with respect to fetal or tumor cells (Neville & Laurence, 1974).

It was the purpose of this study to utilize human colon carcinoma cells established *in vitro* by Tompkins *et al.* (1974) and strains derived from this line to investigate several aspects of CEA at the cell surface. Systems utilizing antibodies specific for CEA were developed to visualize and quantitate this cell surface antigen. The dynamics of antibody-induced redistribution

of CEA were studied and used to determine the relationship of CEA to blood-group A antigen and the time required for CEA to be reexpressed at the tumor cell surface.

Investigations concerning the possible biological significance of CEA on tumorigenicity were conducted in nude mice using cell strains which differed in the amount of CEA they produced.

Further, factors affecting the expression of CEA were examined by establishing inductive systems, in which chemical agents were used to alter the amount of CEA expressed.

Lastly, the clinical significance of CEA was investigated using a specific radioimmunoassay which permitted both the demonstration that no antibodies to CEA could be detected in human sera and the precise quantitation of CEA in serum from cancer patients and controls.

METHODS AND MATERIALS

I. Cell Culture Methods

A. Origin of Human Colon Carcinoma Cell Strains

A strain of human colon tumor cells (HCT-8) was isolated from biopsy material and established in culture by Tompkins *et al.* (1974). This strain was derived from a nonmetastatic adenocarcinoma of the ileocecal region of the colon of a 67 year old male. HCT-8 has previously been well characterized (Tompkins *et al.*, 1974).

Foci of cells with altered morphology were frequently observed during the course of passing these cells *in vitro*. A number of foci were aspirated and removed from the monolayer of HCT-8 cells with a capillary pipette. These were placed in culture and allowed to grow. The aspirated cells were morphologically distinct from the HCT-8 cells. They were subsequently cloned and a selected clone was grown into monolayers which were passaged. These cells were designated HCT-8R. Both these cell strains were used between passages 10 and 50.

B. Additional Cell Strains Used

In addition to HCT-8 and HCT-8R, a strain of human colon carcinoma cells, HT-29, was kindly provided by J. Fogh of the Sloan Kettering Institute, New York, N.Y. Also, a strain of cells established from human rectal carcinoma, HRT-18, was used in the following investigations (Tompkins *et al.*, 1974).

An established strain of human embryonic intestine cells, HEI, was used as controls in CEA studies and obtained from Grand Biological Co. (Grand Island, N.Y.):

Other human cell strains used were Hep-2, carcinoma of the larnyx, KB, epidermoid carcinoma (both obtained from Grand Island Biological Co., Grand Island, N.Y.) and HCF, cultures of colon fibroblasts grown in this laboratory.

C. Maintenance of Cell Cultures

Cultures were grown in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum (FCS), 10 µM/ml of HEPES and tricine buffer, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were grown in sterile plastic screw-cap culture flasks (Corning Glass Works, Corning, New York) at 37°C.

After reaching confluency, cells were passaged by dispersal with a solution containing 0.25% trypsin

and 0.02% ethylenediaminetetracetic acid (EDTA). The monolayer was washed with sterile phosphate buffered saline (PBS) free of calcium and magnesium, 5 to 10 ml of the trypsin-EDTA solution was added and the cultures were placed at 37°C for 5 to 10 minutes or until the cells began to round up and become dislodged from the surface of the culture vessel. After the cells became dislodged an equal volume of fresh medium was added to the flask to inhibit further action of trypsin. The resultant suspension of single cells and small aggregates was then sterilly transferred to a tube and centrifuged at 75 x g for 5 minutes. The supernatant was poured off and the cell pellet was resuspended in fresh medium and approximately 20% of the cells were used to initiate new cultures.

Viable cell counts were obtained by suspending trypsinized cells in a 0.1% solution of trypan blue and counting cells which excluded the dye in a hemocytometer.

The cultures were periodically tested for *Mycoplasma* (Hayflick, 1965) and were consistently found to be free of these organisms.

D. Establishment of Cell Cultures from Nude Mouse Tumors

Select tumors induced in nude mice by injecting HCT-8 or HCT-8R cells were excised and processed for cell culture. After sacrificing the mice, tumors were surgically

removed, debrided of surrounding tissue and minced with a sterile scissors. The minced tissue was washed three times in Hanks balanced salt solution (HBSS) and trypsinized (0.25% trypsin) at 24°C. Dispersed cells were collected after approximately 30 minutes, washed once in HBSS and plated in 25 cm^2 plastic flasks containing RPMI 1640 medium. Fresh medium was added to the cultures 24 hours after plating.

Tumor cell strains which were established from HCT-8 and HCT-8R induced nude mouse tumors were designated HCT-8 Nul and HCT-8R Nul, respectively.

The preceding process was repeated again on selected tumors formed by HCT-8 Nul and HCT-8R Nul, and these cell strains were designated HCT-8 Nu2 and HCT-8R Nu2.

E. Cytogenetic Analysis

For chromosome analysis, Colcemid was added to the culture bottles at a final concentration of 0.1 µg/ml 2 hours before harvesting. The cells were treated with 0.075 M KCl for 30 minutes and fixed in methanol:acetic acid (3:1, v/v). The slides were flame dried and stained with 0.5% Atebrin according to the method of Lin *et al.* (1971). The slides were scanned, and metaphases were photographed with a Zeiss fluorescent microscope under dark-field illumination. The cell cultures under study

were coded, and the karyotypic analyses were recorded before the code was broken. Between 5 and 10 karyotypes were made from each of the cultures, and an additional 5 to 10 photographs of metaphases were analyzed for marker chromosomes.

F. Isolation of Single Cell Clones

The cells to be cloned were dispersed with trypsin EDTA to yield a suspension of separate single cells. They were then pelleted by centrifugation at 75 x g for 5 minutes and resuspended in complete RPMI 1640 growth medium. An aliquot of this suspension was removed and placed in a hemocytometer to obtain a viable cell count. The cell suspension was diluted with medium to yield a final concentration of 5 cells per milliliter. 0.1 ml was then dispersed under aseptic conditions into wells of a sterile Microtest II tissue culture plate (Falcon Plastics, Los Angeles, Calif.). Shortly after plating, the wells of the microplate were inspected with an inverted microscope to determine which wells contained only one cell and these wells were identified with a mark on the overlying lid.

The microplate was incubated in a moist atmosphere containing 5% CO_2 at 37°C. After approximately 10 days, wells containing single colonies were treated with

trypsin-EDTA and the dispersed cells were transfered to sterile, medium containing cell culture tubes. The cells were progressively grown in larger culture vessels, thereby establishing cultures derived from a single cell.

II. *In Vivo* Biological Methods

A. Tumorigenicity in Nude Mice

Cultures of the various cell strains were trypsinized, washed and resuspended in Tris buffered saline (TBS). The cells were enumerated and their viability was assessed by trypan blue dye exclusion. Homozygous (nu/nu) nude RNC mice were obtained from Dr. P. Miniats of Guelph University (Guelph, Ontario, Canada). Groups of 5 nude mice, 5 to 8 weeks old, were given subcutaneous injections, on the back, of various doses of cells ranging from 10^2 to 10^7 cells in 0.1 ml of TBS. The mice were maintained in separate quarters under conventional conditions and observed daily for 2 months for tumor formation. Select tumors were excised and processed for either cell culturing or histological examination. For histology, portions of tumors were fixed in 10% buffered formalin, processed and embedded routinely. Five µm sections were stained by standard hematoxylin-eosin and periodic acid-Schiff techniques. Approximately 40% of the animals bearing tumors were autopsied for metastasis.

III. Immunological Methods

A. Antiserum

Anti-CEA was obtained from Drs. J.L. Palmer and W.A.F. Tompkins (College of Veterinary Medicine, University of Illinois, Urbana, Ill.). It was produced in a goat (#842) by repeated subcutaneous injections of purified CEA emulsified in complete Freund's adjuvant. The CEA for immunization was extracted from liver metastases obtained from a patient with adenocarcinoma of the colon by the method of Krupey *et al.* (1972). The blood type of the patient was O, Rh^+. Anti-CEA produced in a horse was also obtained through the generosity of Dr. P. Gold, McGill University, Montreal, Quebec, Canada. A 1:4 dilution of antisera was absorbed with 50 mg/ml of lyophilized normal human colon mucosa. Adsorption was performed at 37°C for 30 minutes then overnight at 4°C and the serum was then clarified by centrifugation at 2000 x g for 20 minutes followed by centrifugation at 10,000 for 30 minutes. Absorbed antisera were shown by Ouchterlony double diffusion and specific inhibition of cell surface binding with standard reference sources of CEA to be specific for CEA.

Normal goat serum, used as a control, was obtained from non-immunized goats through the courtesy of Dr. J. Gauldie (McMaster University, Hamilton, Ontario, Canada).

Sera from normal individuals of known ABO blood types were used for the studies with isoantigen A. In addition, IgG anti-A was obtained through the courtesy of Dr. J. Bienenstock (Department of Medicine, McMaster University, Hamilton, Ontario, Canada). The anti-A IgG was purified by passing human colostrum through an immunoadsorbant column (Bienenstock & Strauss, 1970). Specificity of the anti-A IgG was demonstrated by removal of antibodies through adsorption with type A red blood cells.

B. Purification of Immunoglobulin G

Immunoglobulin G (IgG) was separated from goat anti-CEA and normal goat serum (NGS) by the method described by Cherry (1974). Serum was initially fractionated by ammonium sulfate $(NH_4)_2SO_4$ precipitation. An equal volume of 60% $(NH_4)_2SO_4$ was added to each serum. Globulin was allowed to precipitate and subsequently packed by centrifugation at 4°C for 30 minutes at 1,440 x g. Globulin was resuspended in distilled water and reprecipitated with an equal volume of 90% $(NH_4)_2SO_4$. The packed, precipitated globulins were again resuspended and extensively dialyzed against 0.01 M sodium phosphate buffer, pH 7.6. The IgG was further purified by DEAE-cellulose 52 column chromatography (Whatman Biochemicals, Kent, England).

The protein concentrations of the IgG-containing fractions were determined by the method of Lowry *et al.* (1951).

C. Fluorescent-Conjugated Immunoglobulins

Anti-CEA IgG was conjugated with fluorescein isothiocyanate as described in Cherry (1974). Unreacted fluorescein was removed by passage through a Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden). Fluorescein isothiocyanate-conjugated rabbit IgG prepared against goat or horse immunoglobulins was obtained from Cappel Laboratories (Downingtown, Pa.). Rhodamine conjugated goat anti-human immunoglobulins were obtained from the same source.

D. Iodine-125 Labeling of Immunoglobulins

IgG purified from goat anti-CEA and NGS were each diluted to 1 mg/ml. Twenty-five µl of each were separately iodinated by the chloramine-T method (McConahey & Dixon, 1966). The radioactive iodine (^{125}I) was obtained from New England Nuclear, Ltd. (Lachine, Quebec, Canada). One mCi of ^{125}I was reacted with 25 µl of IgG in a siliconized tube in the presence of chloramine-T for one minute. Free ^{125}I was separated from ^{125}I-labeled IgG by Sephadex G-25 column chromatography (Pharmacia

Fine Chemicals, Uppsala, Sweden). Fractions were collected in siliconized, albuminized tubes. Labeled fractions were determined by counting a sample of each fraction in an automated gamma counter (Beckman Instruments, Irvine, Calif.). The ^{125}I-labeled IgG was used within 24 hours of preparation and trichloroacetic acid (TCA) precipitation of pooled labeled IgG demonstrated that greater than 85% of counts were bound to IgG.

E. Direct and Indirect Fluorescent Antibody Tests

Cultures of cells to be tested were incubated for 3 days after seeding and the culture fluids replenished with fresh medium one day before testing. The cells were washed in phosphate-buffered saline (PBS) free of calcium and magnesium and dispersed with a solution containing 0.25% trypsin and 0.02% ethylenediaminetetra-acetic acid (EDTA). The cell suspension was washed with medium and resuspended in Tris-buffered saline (TBS) containing 2% FCS. Viability tests using trypan blue uptake showed that 95% or more of the cells were viable. One x 10^5 cells were incubated in 0.05 ml of serum to be tested for 30 minutes at 37°C. Following three washes in TBS, the cells were incubated with 0.05 ml of the appropriate fluorescein or rhodamine-conjugated antisera (diluted 1:10 or 1:20 in TBS) and incubated for an

additional 30 minutes at 4°C. After the second incubation the cells were washed four times in TBS, resuspended in two drops of TBS-glycerol (1:9), and mounted under a glass coverslip. The cells were examined for fluorescence with a Leitz OrthoPlan Microscope. For fluorescein isothiocyanate, excitation filters BG 38 and BG 12 were used with a dichroic mirror TK 495 combined with K 495 and K 510 barrier filters. For rhodamine, the BG 38 and A 1546 excitation filters were used with a TK 580 dichroic mirror and K 490 barrier filter. Serum controls consisted of normal goat serum in the case of anti-CEA studies and serum from patients with blood types A or AB in the case of anti-A studies.

The direct fluorescence antibody test was performed by incubating fluorescein isothiocyanate-conjugated anti-CEA antibodies with test cells for 30 minutes at 37°C after which the cells were washed four times in TBS, resuspended in TBS-glycerol, and mounted. Controls consisted of cells incubated with fluorescein labeled normal goat IgG.

F. Direct and Indirect Iodine-125 Labeled Immunoglobulin Tests

Cell surface CEA was quantitated by assaying the direct binding of ^{125}I-anti-CEA IgG. The parameters of this reaction were determined empirically (see Results).

Cells from 3-day-old cultures were monodispersed with trypsin-EDTA, washed with TBS and resuspended in TBS at a concentration of 5 x 10^6 cells/ml. Cells were aliquoted into siliconized 12 x 75 mm glass test tubes at 1 ml/tube. The cells were pelleted by centrifugation at 75 x g for 5 minutes and the supernatants were decanted. To the cells were added 50 µl of either ^{125}I-labeled IgG from NGS or ^{125}I-anti-CEA IgG. Antibody was allowed to react for 45 minutes at 24°C after which the cells were washed five times with TBS, transferred to a new test tube, and counted in an automatic gamma counter. All assays were performed in duplicate and specific binding was calculated by subtracting the average counts per minute (cpm) of ^{125}I-NG IgG from the average cpm of ^{125}I-anti-CEA IgG.

A similar indirect assay system employing ^{125}I-labeled rabbit anti-goat (RAG) IgG, was established to quantitate cell surface CEA and further to determine the fate of antibodies bound to cell surface CEA. The optimal conditions for the indirect assay were again determined empirically (see Results). In general, 0.5-1 x 10^6 cells were aliquoted into siliconized 12 x 75 mm glass test tubes. Cells were pelleted and reacted for 30 minutes at 24°C with a 1:20 dilution of goat anti-CEA serum or a 1:20 dilution of normal goat serum (NGS) as a control. The cells were then washed three

times with TBS to remove unbound antibodies. After pelleting the cells, they were then reacted with 50 µl of ^{125}I-RAG IgG for 30 minutes at 24°C. Unbound counts were removed by washing the cells five times with TBS. The cells were then transferred to a new tube and counted in an automatic gamma counter.

All reactions were carried out in duplicate and specific cmp were calculated by subtracting counts bound to cells reacted with NGS from counts bound to cells reacted with goat anti-CEA antiserum.

G. Other Methods of Determining Cellular Carcinoembryonic Antigen Production

Production of CEA by the various cell strains was also determined by assaying the supernatants from 8-day-old cultures. The culture medium was collected, centrifuged at 75 x g for 10 minutes to remove cell debris and stored at -20°C until tested. Under code, the CEA content of supernatants was measured by the Z-gel method of LeGerfo *et al.* (1971) with reagents obtained from Hoffmann-LaRoche, Inc. (Nutley, N.J.). These tests were kindly conducted in the laboratory of Dr. P. Dent.

IV. Methods of Determining the Cell Surface Dynamics of Carcinoembryonic Antigen

A. Inhibitor Studies of Carcinoembryonic Antigen Cell Surface Redistribution

The effect of sodium azide (Eastman Kodak, Rochester, N.Y.) cycloheximide (Sigma Chemical Co., St. Louis, Mo.), cytochalasin B (Aldrich, Milwaukee, Wis.) and colchicine (Sigma Chemical Co., St. Louis, Mo.) on antibody-induced capping was studied as follows: 1×10^6 cells were incubated for 15 or 60 minutes at 37°C in 2 ml of phosphate buffered saline containing 2% FCS and a known concentration of inhibitor. Following incubation of cells with the inhibitors the indirect immunofluorescence test for CEA was carried out at 37°C as described above, with the exception that the immunologic reagents and washing reagents contained the appropriate concentration of inhibitor being tested. Cells were observed for antibody-induced cap formation in the presence and in the absence of inhibitor and the percent inhibition of capping was calculated.

B. Determining the Fate of Antigen-Antibody Complexes at the Cell Surface

Radiolabeled rabbit anti-goat (RAG) IgG was used to determine whether CEA-anti-CEA complexes at the cell surface were shed or endocytosed after lateral redistribution. One x 10^6 HCT-8R cells were incubated with goat anti-CEA or normal goat serum as previously described. Unbound antibodies were removed by washing the cells in PBS. The tubes were subsequently divided into three groups. The first group of tubes immediately received 50 µl of RAG-I^{125} which was incubated with the cells for 30 minutes at 24°C. Unbound counts were removed by washing. Tubes from this group were then either immediately counted, to establish the maximum specific cpm at time 0, or were incubated for various lengths of time at 37°C. Incubating cells which had already reacted with RAG-I^{125} at 37°C would permit determining whether the CEA-anti-CEA complexes at the cell surface were endocytosed or shed. As additional controls, a second group of tubes was reacted with RAG-I^{125} as above, but tubes were then placed at 0°C for various lengths of time. This procedure was shown to inhibit lateral redistribution of CEA-anti-CEA complexes at the cell surface. The third group of tubes was incubated at 37°C for various lengths of time after reacting the

cells with goat anti-CEA or NGS. Only after various time periods at 37°C were these cells reacted with RAG-I^{125}. In this manner, loss of CEA-anti-CEA complexes from the cell surface by either endocytosis or shedding could be quantitated. All reactions were performed in duplicate and specific cpm was calculated by subtracting counts of RAG-I^{125} bound to cells reacted with NGS from cells reacted with goat anti-CEA antiserum.

C. Determining the Turnover Time of Carcinoembryonic Antigen at the Cell Surface

The time required for the reexpression of CEA at the cell surface after antibody-induced redistribution was quantitated with the direct ^{125}I-labeled immunoglobulin test. Five x 10^6 HCT-8R cells were preincubated for 30 minutes at 37°C in PBS or PBS containing 20 µg/ml of cycloheximide. A set of tubes from each of these groups were then reacted with ^{125}I-anti-CEA IgG or ^{125}I-normal goat IgG to obtain initial specific cpm, i.e. quantity of cell surface CEA at the beginning of the experiment. The rest of the tubes received 75 µl of a 1:16 dilution of unlabeled goat anti-CEA antiserum. This was reacted with the cells for 30 minutes at 24°C and unbound antibodies were removed by washing the cells with PBS. The tubes were then divided into three groups.

The first group was incubated in PBS at 37°C for various lengths of time followed by reaction with ^{125}I-anti-CEA IgG or ^{125}I-normal goat IgG. Since the unlabeled antibodies inhibit the binding of iodine-labeled antibodies, this procedure should permit quantitation of newly synthesized cell surface CEA. As additional controls, the second group of tubes was incubated in PBS containing 20 µg/ml of cycloheximide at 37°C for various lengths of time before reaction with radiolabeled IgG. Incubation of tubes in this manner should permit a determination of the requirement for protein synthesis. The third group of tubes were incubated at 0°C for various time periods before incubation with radiolabeled IgG. Since low temperature was shown to inhibit antibody-induced redistribution of CEA, this condition should completely inhibit the turnover of cell surface CEA. All determinations were made in duplicate and specific cpm was calculated by subtracting the average cpm of cells reacted with ^{125}I-normal goat IgG from average cpm of cells reacted with ^{125}I-anti-CEA IgG.

D. Comparison of Isoantigen A and Carcinoembryonic Antigen on the Cell Surface

One x 10^6 HT-29 cells, which express both CEA and blood group A antigens, were reacted with fluorescein

conjugated anti-CEA for 60 minutes at 37°C. The cells were washed and fixed with 2.5% buffered formalin for 4 minutes. Cells were then reacted with specific antibodies to isoantigen A or IgM anti-A antibodies for 30 minutes at 4°C, washed and reacted with rhodamine-conjugated goat anti-human globulin. The same reactions were also performed in reverse, i.e. anti-A antibodies were bound to HT-29 cells before the addition of fluorescein-labeled anti-CEA antibodies.

Counts were made by first identifying a cell with polar distributed CEA, and viewing that cell for rhodamine-staining distribution by altering the microscope filters.

V. Methods of Determining Factors Which Affect the Surface Expression of Carcinoembryonic Antigen

A. Inducing Agents

A number of chemicals previously shown to affect the synthesis of "differentiation-associated" gene products were examined for their effect on CEA synthesis. These consisted of: dimethylsulphoxide (DMSO) (Fisher Scientific Co., Fairlawn, N.J.), dexamethazone (Organon Canada Ltd., Toronto, Ont.), bromodeoxyruidine (BrdU) (Sigma Chemical Co., St. Louis, Mo.), theophylline (Sigma

Chemical Co., St. Louis, Mo.) and dibutyryl cyclic adenosine monophosphate (dbcAMP) (Sigma Chemical Co., St. Louis, Mo.).

B. Screening for Inductive Effects

Cultures of HEI, HCT-8 Nu2, HCT-8 and HCT-8R were each seeded with approximately 1 x 10^7 cells on day 0. Cells were allowed 24 hours to enter log phase growth at which time medium was replaced with medium containing inducing agent. The following concentrations of inducing agents were used: 10 µg/ml of dexamethazone, 1.5% (v/v) DMSO, 1 mM theophylline, and 3 µg/ml of BrdU. Cultures incubated with BrdU were wrapped in foil to prevent exposure to light and all cultures were incubated for various lengths of time at 37°C.

After incubation, cultures were trypsinized and the quantity of cell surface CEA was determined by the direct radiolabeled immunoglobulin test. The quantity of CEA present in induced cells was compared to the quantities of CEA present on an equal number of control cells incubated in normal medium. All tests were conducted in duplicate.

The ratio of induction was calculated by dividing the specific cpm of induced cultures by the specific cpm of control cultures then subtracting one from this number.

VI. Seroepidemiologic Methods

A. Detection of Antibodies to Carcinoembryonic Antigen in Patient Serum

The ability of unlabeled anti-CEA antibodies to specifically block the binding of ^{125}I-anti-CEA IgG to cell surface CEA was used to detect antibodies to CEA in human sera.

Fourteen sera obtained from healthy laboratory personnel and 20 sera obtained from patients with colorectal carcinoma were diluted 1:5 with TBS and incubated with 5×10^6 HCT-8R cells for 45 minutes at 24°C. As controls, a 1:100 dilution of goat anti-CEA antiserum or TBS was similarly incubated. Following incubation, unbound antibodies were removed by washing and the cells were reacted separately with ^{125}I-anti-CEA IgG or ^{125}I-normal goat IgG in the direct cell surface CEA assay. Specific cpm were calculated, the maximum being represented by control cells incubated in TBS.

B. Serum Panels Examined for Carcinoembryonic Antigen Content

A series of studies were conducted using various serum panels derived from patients with and without cancer to determine the relative diagnostic applicability of

our human colon tumor cell solid-phase inhibition radioimmunoassay (RIA). Twenty sera were obtained from patients with colo-rectal cancer, these sera were examined in conjuction with sera obtained from 25 normal healthy individuals. A second test consisting of 13 sera obtained from patients with cervical cancer were compared to 13 age, sex and race matched controls. Thirteen sera from patients with endometrial cancer were examined with 13 sera obtained from women with ovarian cancer. Sixteen sera from breast cancer patients and 12 sera from lung cancer patients were examined, and 20 sera from patients with hepatitis B or non-B hepatitis were examined.

Standard reference CEA obtained from Hoffmann-LaRoche (Nutley N.J.) and an international CEA standard (National Institute for Biological Standards, London, Eng.) were used to standardize our assay system. It should be noted that the activity of the international standard was given in units with one unit present in 0.0236 mg of freeze-dried powder.

C. Comparison of our Radioimmunoassay with the Hoffmann-LaRoche Assay

Thirteen breast cancer sera and 11 lung cancer sera were provided to this laboratory for quantitation of CEA level under code from Dr. P. Dent (McMaster University,

Hamilton, Ont.). Previously, Dr. P. Dent had quantitated the level of CEA in the same patients' plasma using the Z-gel method of LoGerfo *et al.* (1971).

After conducting our assay on these sera, the code was broken and results were compared using linear regression analysis.

D. Human Colon Tumor Cell Solid-Phase Inhibition Radioimmunoassay for Carcinoembryonic Antigen

Accurate quantitation of CEA in patient sera could be determined by the degree of inhibition of binding of ^{125}I-anti-CEA to CEA present on the surface of human colon carcinoma cells grown *in vitro*.

For this assay, 125 µl of patient serum or TBS were separately incubated for 2 hours at 37°C with 50 µl of ^{125}I-anti-CEA IgG and ^{125}I-normal goat IgG. After this period, 5×10^6 HCT-8R cells in 100 µl of TBS were added and incubated at 24°C for 45 minutes. Counts not bound to the cells were removed with five washes. The cells were then transferred to a fresh tube, pelleted and counted in a gamma counter.

All determinations were made in duplicate. Specific cpm was calculated by subtracting average cpm bound to cells reacted with ^{125}I-normal goat IgG from average counts bound to cells reacted with ^{125}I-anti-CEA

IgG. Maximum specific cpm were calculated from cells incubated with TBS in place of patient serum, i.e. no CEA to inhibit binding and this value was taken as 0% inhibition of binding.

A serum sample obtained from a healthy donor (L.G.) was consistently run in each test. All serological studies were calculated relative to this serum and the inter-test variation obtained was used to determine a "normal" threshold level of CEA.

RESULTS

I. Test Systems: Determination of Optimal Reaction Conditions and Specificity

A. Antiserum

Carcinoembryonic antigen (CEA), as a molecular species, has been defined immunologically. Consequently, preparations of CEA used in various studies require demonstration of antigenic identity to standard reference sources of CEA. The antiserum used in the following studies was shown by Ouchterlony double diffusion to be specific for a CEA preparation and a line of identity was obtained between our anti-CEA and anti-CEA obtained from Dr. P. Gold (Fig. 1).

Initially this antiserum was used in both direct and indirect immunofluorescent studies of cell surface CEA. To further demonstrate specificity of our antiserum to CEA, a number of different cultured cell strains derived from malignant and normal tissues were examined. The results, shown in Table 1, indicate that three cell lines, HCT-8, HRT-18 and HT-29, which were derived from human

Figure 1

Photograph of immunodiffusion slide of CEA and normal colon extract (NCE) reacted against horse anti-CEA obtained from Dr. P. Gold (A-CEA Gold) and goat anti-CEA (842). A line of identity was obtained with the two antisera against CEA.

842

NCE

CEA

A-CEA GOLD

Table 1

Reaction of anti-CEA with Cells Derived from Different Human Tissues

Cell	Tissue origin	Immunofluorescence reaction with anti-CEA serum[a]	Immunofluorescence reaction with normal goat serum[b]
HCT-8	Adenocarcinoma colon	+	-
HRT-18	Adenocarcinoma of rectum	+	-
HT-29	Adenocarcinoma colon	+	-
HEI-407	Embryonic intestine	-	-
KB	Epidermoid carcinoma	-	-
Hep-2	Carcinoma of larnyx	-	-
HCF	Colon fibroblast	-	-

[a] A 1:20 dilution of goat anti-CEA which had been adsorbed with normal colon mucosa was added to viable cells in suspension and the indirect immunofluorescence test carried out.

[b] Similar preparation of normal goat serum.

intestinal carcinomas reacted with anti-CEA but not with normal goat serum while cells derived from malignancies of other sites or from normal tissues did not react with either serum. Further, the cell lines which reacted by immunofluorescence have previously been shown by radioimmunoassay to synthesize CEA (Tompkins *et al.*, 1974; Egan & Todd, 1972).

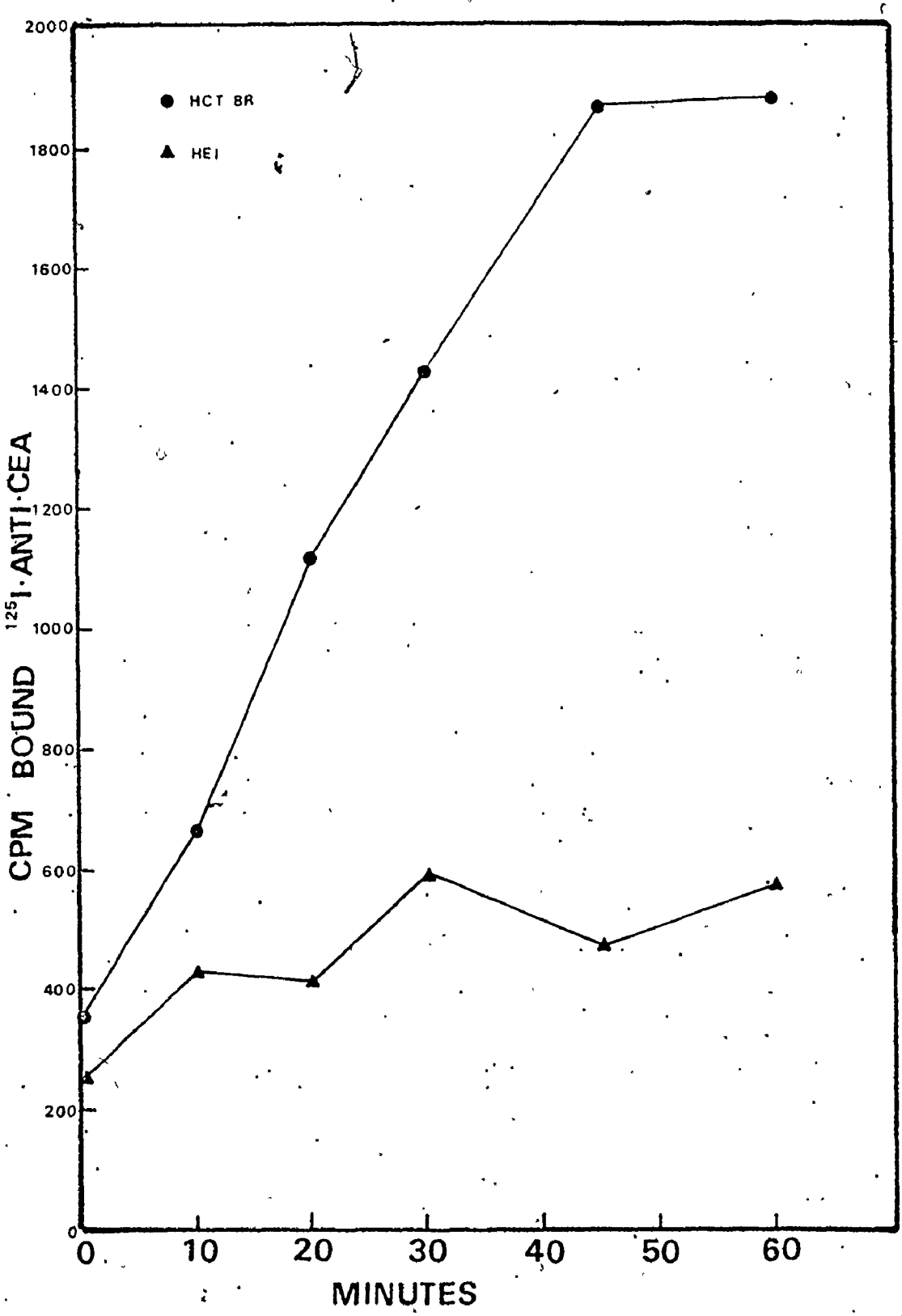
While immunofluorescence techniques provide the ability to directly visualize the topography of antigen on a cell and permit an approximate determination of the degree of expression and percentage of cells in a population expressing the antigen, immunofluorescence does not provide precise quantitation. Therefore, direct and indirect systems utilizing iodine-125 labeled antibodies were developed.

B. Direct Radioimmunoassay for Cell Surface Carcinoembryonic Antigen

The direct reaction was optimized by varying each parameter of the test separately while holding the others constant. The parameters examined were time, temperature, cell number and dilution of radiolabeled antibody. As can be seen in Figure 2, maximum binding of ^{125}I-anti-CEA was achieved after 45 minutes at 24°C. Similar time was required for maximum binding of labeled antibody at 37°C

Figure 2

Time required to achieve maximum binding of ^{125}I-anti-CEA. 1 x 10^6 HEI (▲) or HCT-8R (●) cells were incubated with 100 µl of ^{125}I-anti-CEA for various lengths of time at 24°C. Unbound counts were removed by washing and bound counts per minute (cpm) were determined by counting cell pellets in the gamma counter. Each point represents the average of duplicate tubes.


HCT 8R
HEI
CPM BOUND ^{125}I·ANTI·CEA
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
MINUTES

but over the short time period examined maximum binding was never achieved with incubation at 4°C. Specificity of the reaction was indicated by the marked increase of binding to HCT-8R with time while binding to HEl, a non-CEA producing line, remained fairly constant over time and at background levels.

During these initial experiments, samples of the washes were examined and it was determined that four washes were required to remove non-specific labeled antibody from the reaction mixture. Subsequently, five washes were routinely used to insure complete removal of non-specific unbound antibody.

Additional studies concerned with optimizing conditions of the direct reaction indicated (Figure 3) that the ability to detect CEA was rapidly diminished by diluting the radiolabeled antibody. While a marked difference between HCT-8R and HEl cells were seen using undiluted ^{125}I-anti-CEA. This difference was abrogated by a two-fold dilution.

Similar studies indicated (Figure 4) that specific binding of anti-CEA increased with increasing numbers of HCT-8R cells. Specific binding to HEl cells, on the other hand, did not appreciably increase with increasing cell numbers.

From these data, standard optimal conditions for the direct reaction were derived and these involved

Figure 3

Effect of dilution of ^{125}I-anti-CEA on the detection of cell surface carcinoembryonic antigen. 100 µl of various dilutions of ^{125}I-anti-CEA were incubated with 1 x 10^6 HEI (Δ) or HCT-8R (●) cells for 45 minutes at 24°C. Unbound counts were removed by washing and bound counts per minute (cpm) were determined by counting cell pellets in the gamma counter. Each point represents the average of duplicate tubes.

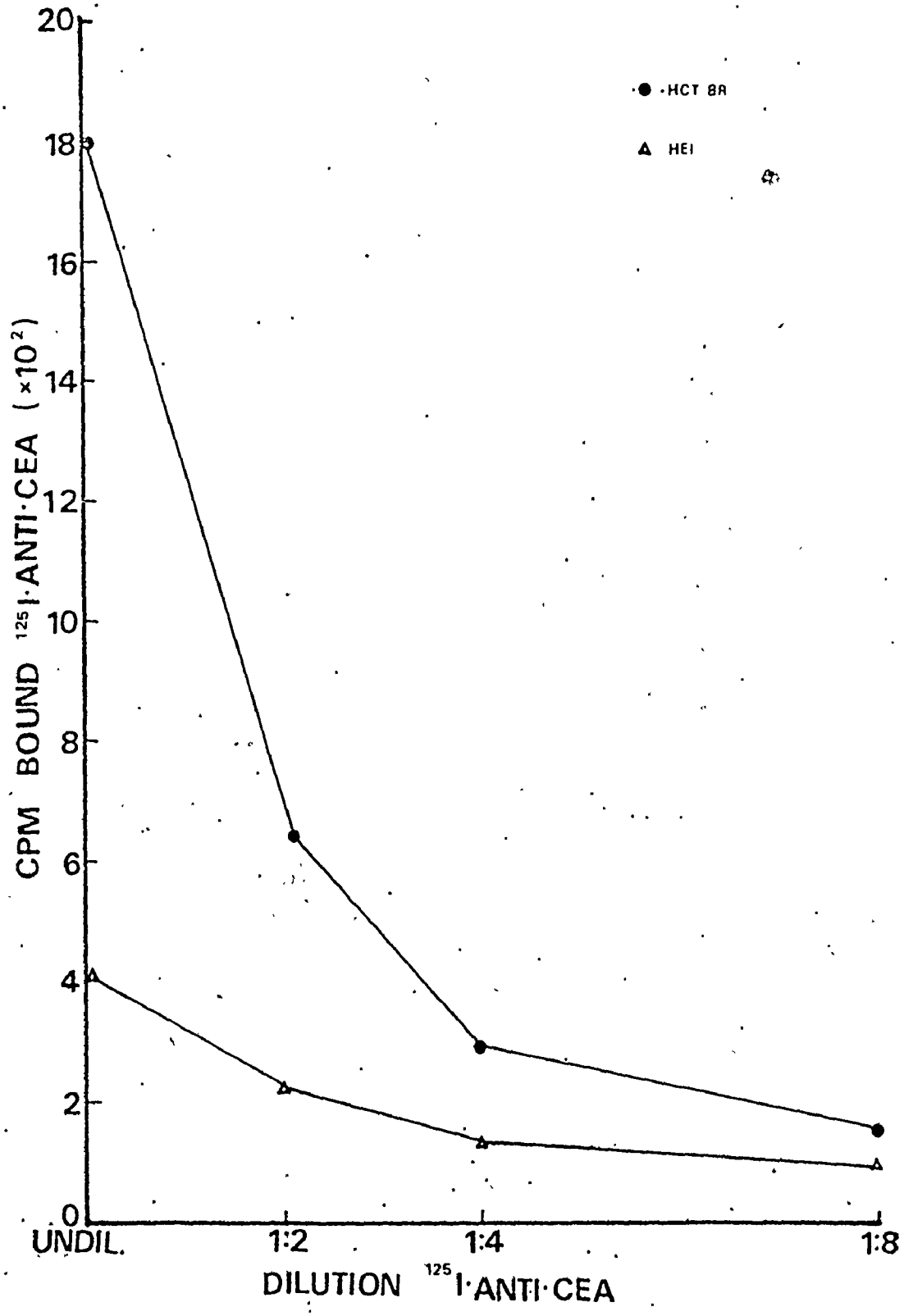
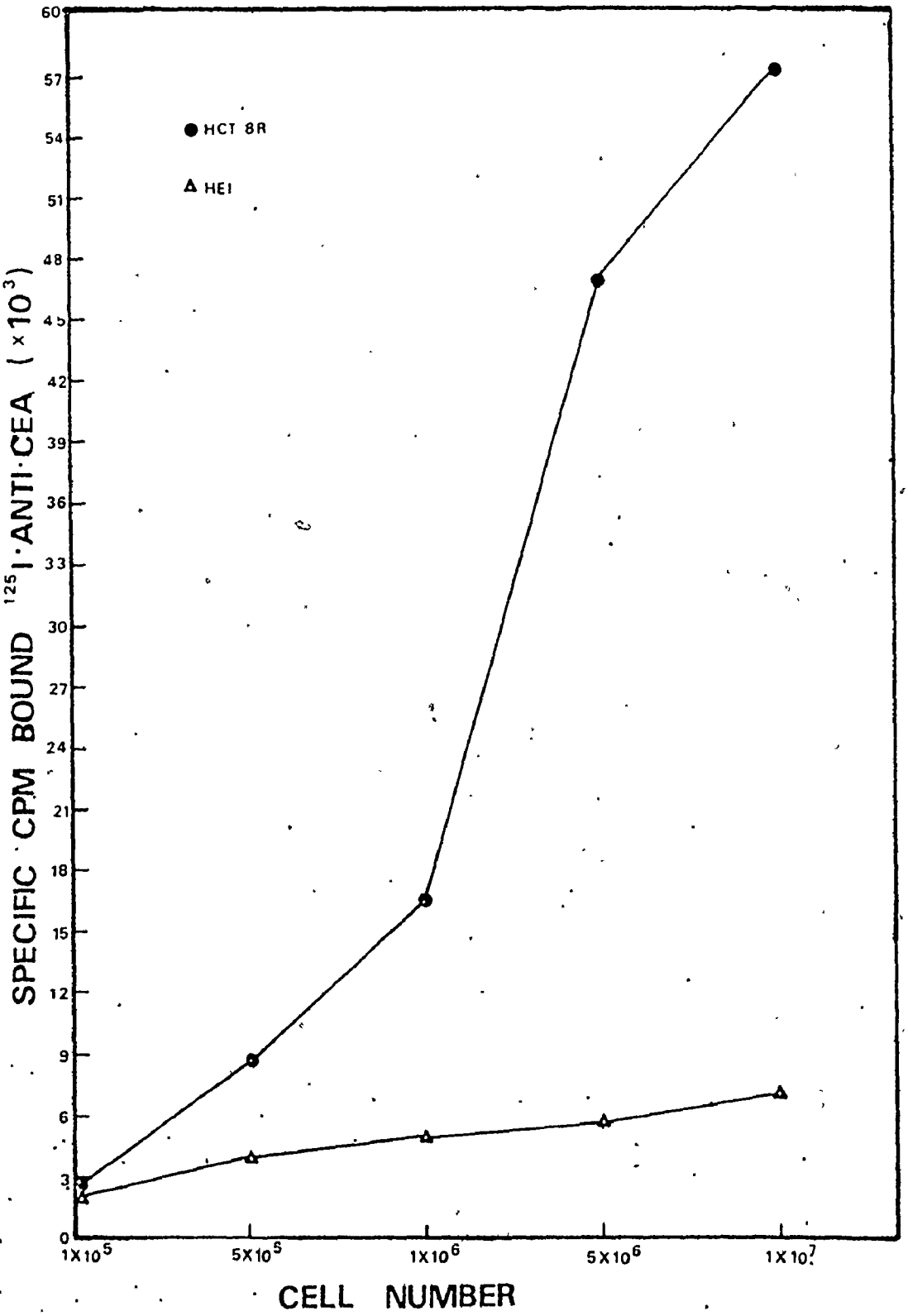

20
18
16
14
12
10
8
6
4
2
0
CPM BOUND ^{125}I·ANTI·CEA (×10^2)
HCT 8R
HEI
UNDIL.
1:2
1:4
1:8
DILUTION ^{125}I·ANTI·CEA

Figure 4

Effect of various cell numbers on specific binding of ^{125}I-anti-CEA. Various numbers of trypsinized, viable HEI (Δ) or HCT-8R (●) cells were separately incubated with 50 µl of ^{125}I-anti-CEA or ^{125}I-normal goat IgG for 45 minutes at 24°C. Unbound counts were removed by washing and bound counts were determined by counting cell pellets in the gamma counter. Specific counts per minute (cpm) were determined by subtracting the average counts bound to cells from duplicate tubes incubated with ^{125}I-normal goat IgG from the average counts obtained from duplicate tubes incubated with ^{125}I-anti-CEA.


HCT 8R
HEI
SPECIFIC CPM BOUND ^{125}I·ANTI·CEA ($\times 10^3$)
CELL NUMBER
1X10^5
5X10^5
1X10^6
5X10^6
1X10^7
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54
57
60

the binding of undiluted radiolabeled antibody for 45 minutes at 37°C or room temperature to approximately 2.5-5 x 10^6 cells.

C. Effects of Trypsinization on Cell Surface Carcinoembryonic Antigen

Since CEA represents a cell surface glycoprotein, the possibility existed that some of it was removed by trypsinization of the cells. That trypsinization did not markedly alter the amount of cell surface CEA was demonstrated in three ways. First, trypsinized cells were incubated in medium or trypsin for one-half hour and then tested in the direct reaction. Results from this experiment indicated comparable levels of ^{125}I-anti-CEA binding to both cell sets. Similarly, when cell surface CEA was quantitated by the direct reaction either immediately after trypsinization or after four hours in suspension at 37°C in complete medium similar counts of bound antibody were obtained. Lastly, comparisons of CEA content on HCT-8R cells obtained after trypsinization from monolayer culture or of samples of HCT-8R cells adapated to suspension culture (compliments of Dr. S. Bacchetti) were identical after adjusting for the increased surface area of the larger suspension culture cells (Table 2).

Table 2

Comparison of CEA Content on Trypsinized Monolayer-derived and Suspension Cultured Cells

Cell source	Specific cpm[a]	Cell diameter[b]	Cell surface area[c]	Specific cpm/ cell surface area
Monolayer	70483	2.0	12.5	5.6×10^3
Suspension	135857	2.5	19.6	6.9×10^3

[a] Specific cpm = average cpm of I^{125}-anti-CEA - average cpm I^{125}-normal goat IgG. Bound to 5×10^6 cells.

[b] Mean of 10 measurements (μm).

[c] $4\pi r^2$ = surface area of a sphere.

D. Specificity of Radioimmunoassay

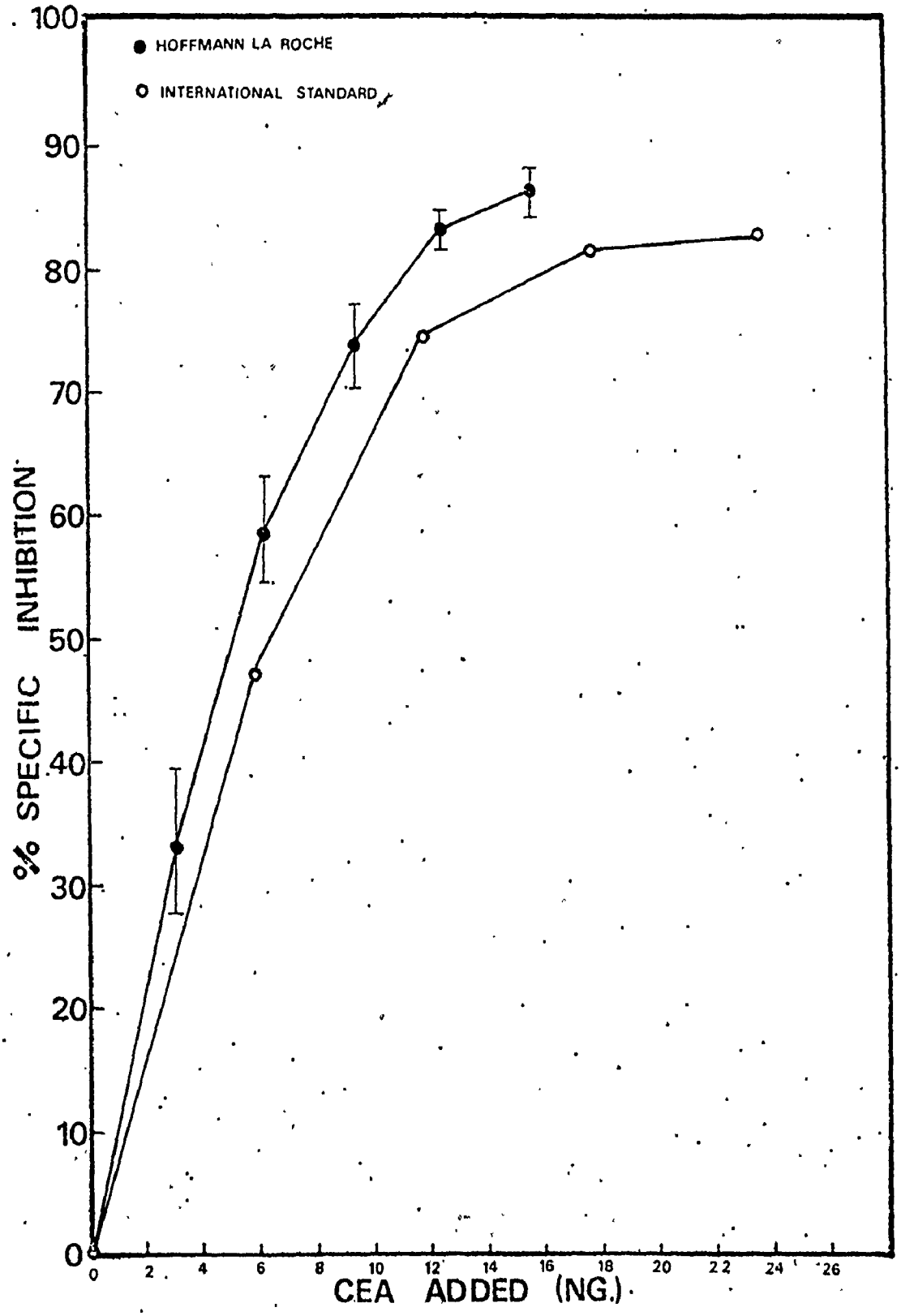
Lastly, specificity of our radioimmunoassay (RIA) was demonstrated using two reference standards of CEA to inhibit the binding of radiolabeled antibody to the cultured colon carcinoma cells. Figure 5 illustrates that increasing quantities of soluble reference CEA produced increasing inhibition of cell surface binding. Six nanograms of both the Hoffmann-LaRoche (kindly provided by Dr. P. Dent) and the International Standard of CEA (obtained from the National Institute for Biological Standards and Control, London, England) were required to inhibit 50% of cell surface binding and each maximally inhibited binding to 86% and 82%, respectively. The fact that no overlap was observed between the two curves may indicate a slight quantitative difference between the two standards and would suggest a slightly higher degree of specificity from the commercially obtained source. Yet both preparations were able to inhibit binding to a great extent, hence, demonstrating a high degree of specificity for the assay.

Assuming the population of cells is homogeneous with respect to the amount of CEA per cell (which is demonstrated later), the inhibition curves can be used to estimate the number of molecules of CEA per cell. Calculations, outlined in Appendix I, indicate that approximately 8×10^3 molecules of CEA are expressed on each HCT-8R cell.

Figure 5

Specificity of direct cell surface carcinoembryonic antigen (CEA) radioimmunoassay demonstrated by inhibition of binding with two standard reference sources of CEA. Constant volumes (125 µl) containing various known quantities of Hoffmann-LaRoche reference CEA (●) or an International Standard of CEA (o) were separately incubated with 50 µl of ^{125}I-anti-CEA or ^{125}I-normal goat IgG in duplicate for two hours at 37°C. Subsequently, 100 µl containing 5×10^6 viable HCT-8R cells were added to each tube. These were incubated at 24°C for 45 minutes after which counts not bound to the cells were removed by washing. Specific counts per minute (cpm) were determined by subtracting the average of counts obtained with ^{125}I-normal goat IgG from counts obtained by binding of ^{125}I-anti-CEA. Percent specific inhibition was calculated from the maximum specific binding obtained from samples incubated without any CEA. Each point represents duplicate determinations and the range of two experiments performed with the Hoffmann-LaRoche standard reference CEA are indicated.

NOTE: Units of international reference CEA were converted to nanograms (ng), 1 unit = 23.6 ng.


HOFFMANN LA ROCHE
INTERNATIONAL STANDARD
% SPECIFIC INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
CEA ADDED (NG.)

E. Indirect Radioimmunoassay for Cell Surface Carcinoembryonic Antigen

The optimal conditions for the indirect radioimmunoassay (RIA) were determined and in this assay iodine-125 labeled rabbit anti-goat IgG (RAG-I^{125}) was employed. It was demonstrated that 30 minutes was required to achieve maximum binding of RAG-I^{125} at 24°C (Figure 6). A similar amount of time was required for maximum binding at 37°C but again incubation at 4°C over the short time period examined never reached levels attained with higher temperatures.

A direct correlation between cell number and specific counts per minute (cpm) was demonstrated (Figure 7) and appeared to reach a maximum with about 1 x 10^6 cells.

The influence of concentrations of primary antibody used in the indirect assay is shown in Figure 8. Specific binding could be detected at a 100,000 dilution of antiserum by this procedure.

Finally, it was shown that approximately 75 µl of RAG-I^{125} were required to obtain maximum detection of specific antibody bound to the cell surface (Figure 9).

Figure 6

Time required to achieve maximum binding of rabbit anti-goat-^{125}I (RAG-I^{125}) in the indirect radioimmunoassay. Fifty µl of 1:16 dilutions of normal goat serum or goat anti-CEA were separately reacted with 1 x 10^6 HCT-8R cells for 30 minutes at 37°C. Unbound antibodies were removed by washing and then each tube was reacted with 100 µl of RAG-I^{125} for various lengths of time at either 24°C (●) or 0°C (o). Unbound counts were removed by washing. Each point plotted represents the average of duplicate determinations. Specific counts per minute (cpm) were calculated by subtracting the average counts bound to cells reacted with normal goat serum from the average counts bound to cells reacted with goat anti-CEA.

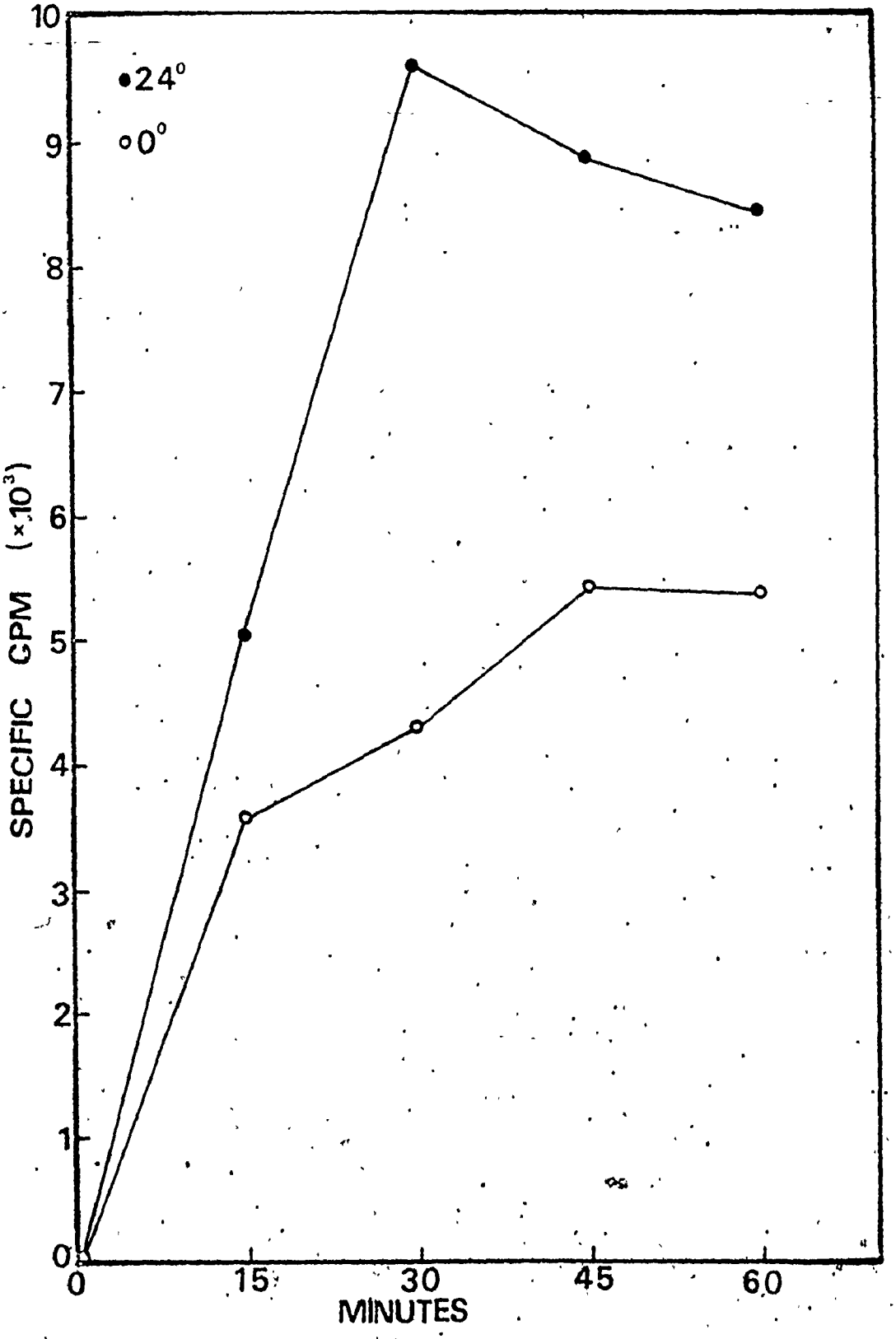

10
9
8
7
6
5
4
3
2
1
0
•24°
∘0°
SPECIFIC CPM (×10³)
0
15
30
45
60
MINUTES

Figure 7

Effect of various cell numbers on specific binding in the indirect radioimmunoassay. Various numbers of trypsinized, viable HCT-8R cells were reacted with 50 µl of 1:16 dilutions of normal goat serum on goat anti-CEA for 30 minutes at 37°C. Unbound antibodies were removed by washing. All tubes were then reacted with 50 µl of rabbit anti-goat-I^{125} (RAG-I^{125}) for 30 minutes at 24°C. Unbound counts were removed by washing. Each point represents the average of duplicate determinations. Percent maximum specific binding was calculated relative to specific counts per minute (cpm) obtained using 1×10^6 cells.

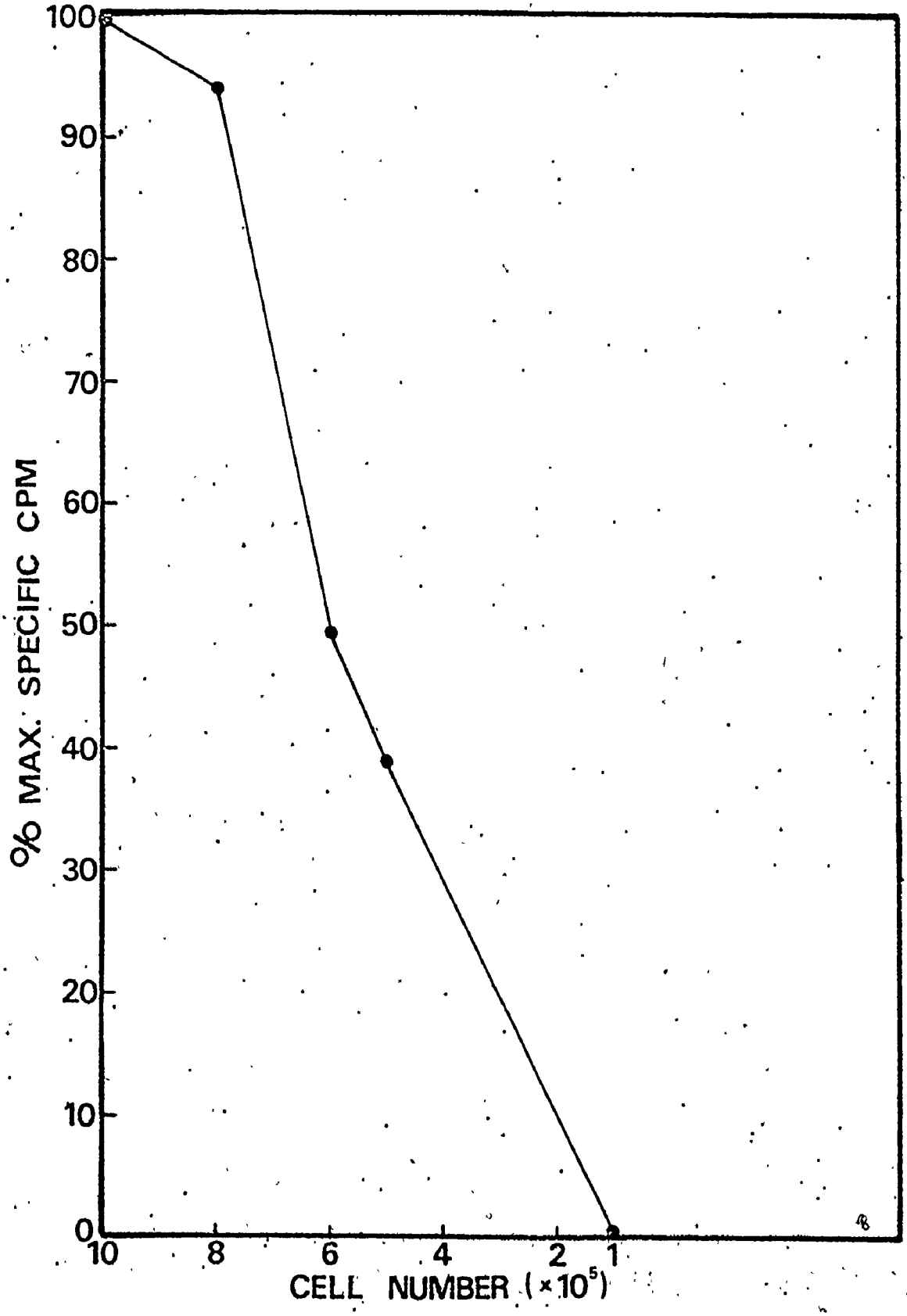

100
90
80
70
60
50
40
30
20
10
0
% MAX. SPECIFIC CPM
10
8
6
4
2
1
CELL NUMBER (×10⁵)

Figure 8

Effect of primary antibody dilution on detection of specific binding by indirect radioimmunoassay. 1 x 10^6 HCT-8R cells were reacted in duplicate with various dilutions of normal goat serum or goat anti-CEA in 50 µl for 30 minutes at 37°C. Unbound antibodies were removed by washing. Subsequently, all tubes were reacted with 50 µl of RAG-I^{125} for 30 minutes at 24°C. Specific counts per minute (cpm) were determined by subtracting the average counts bound to cells reacted with normal goat serum from the average counts bound to cells reacted with goat anti-CEA. Percent maximum binding was calculated relative to specific binding obtained with a 1:32 dilution of anti-CEA.

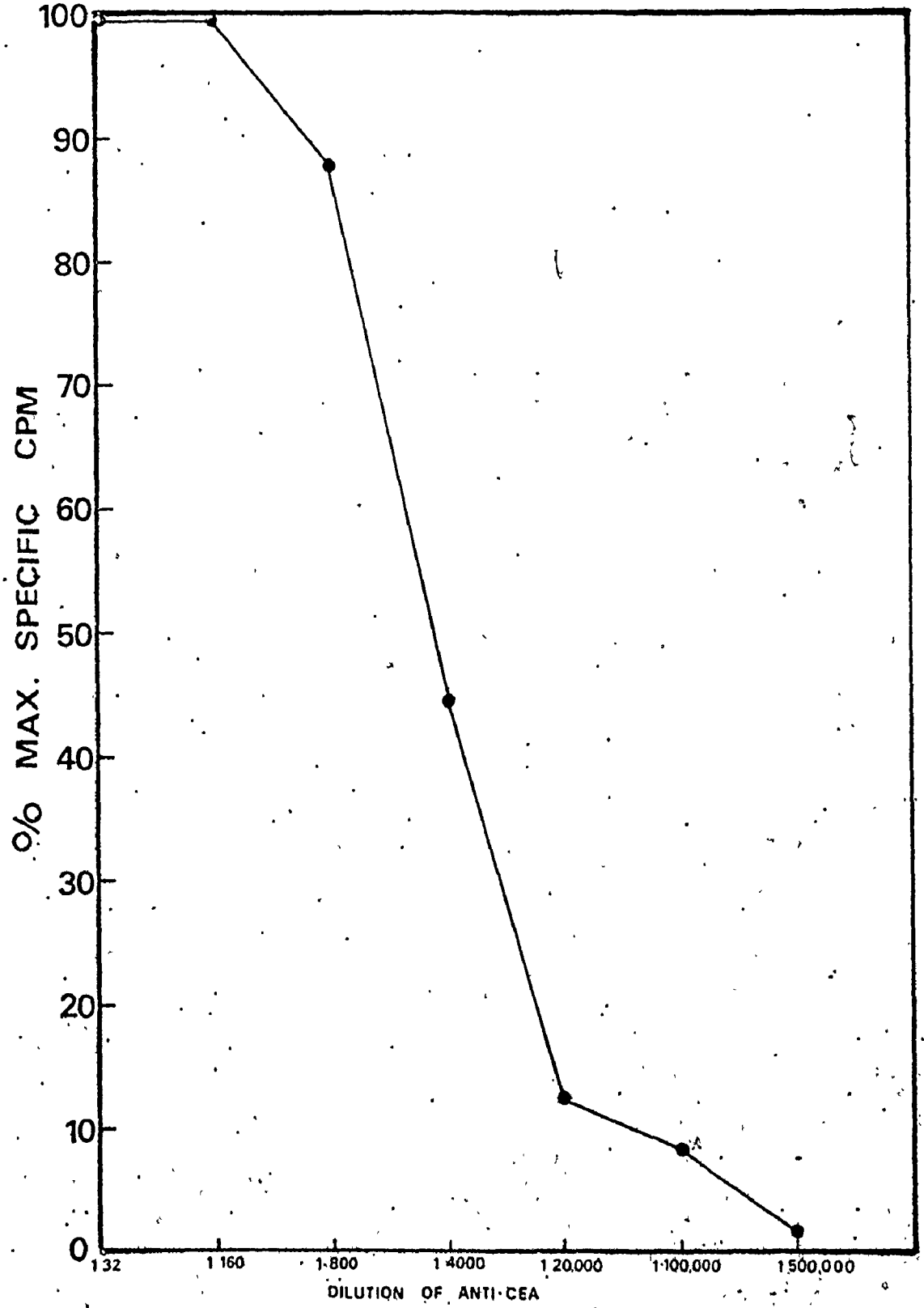
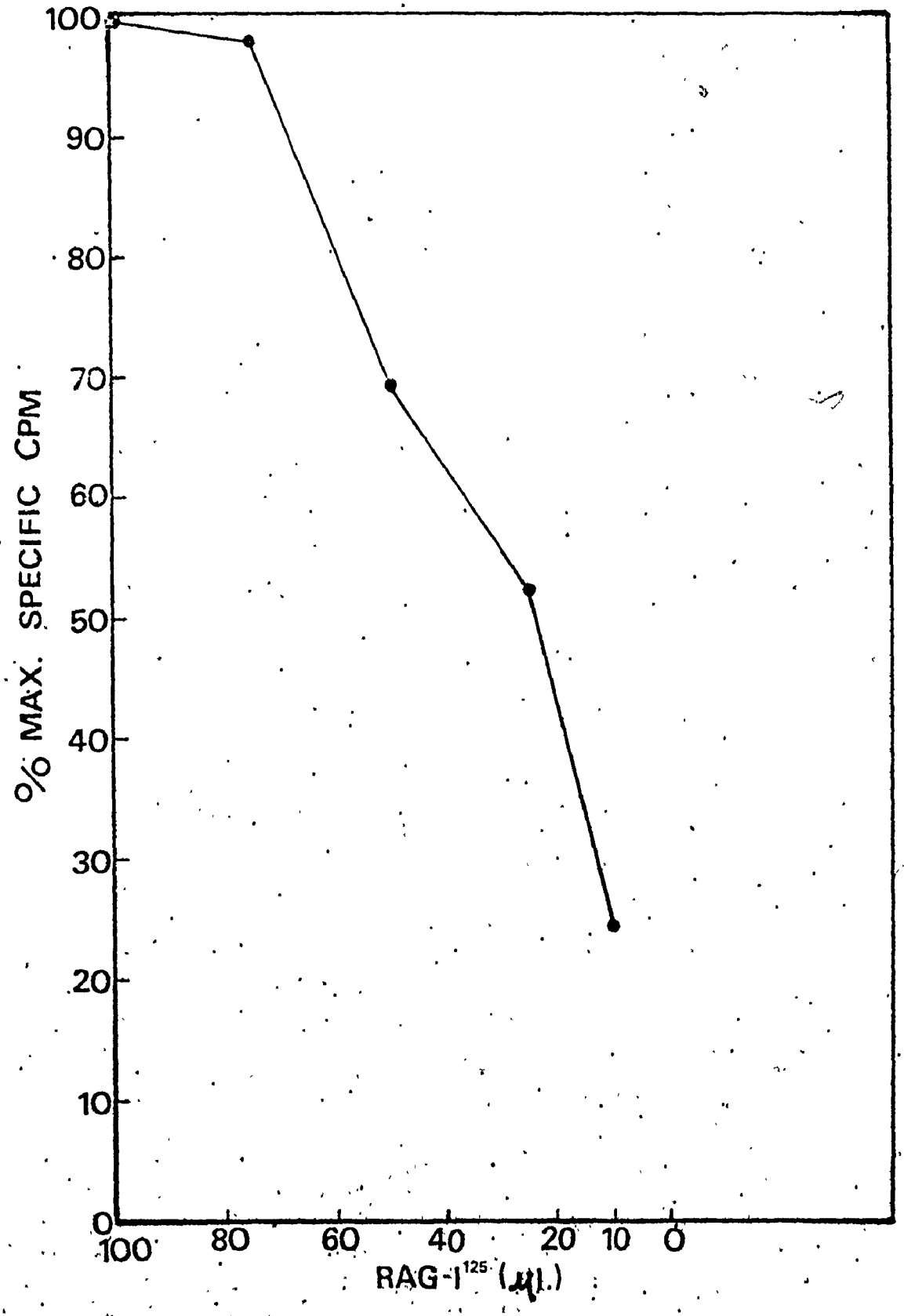

100
90
80
70
60
50
40
30
20
10
0
% MAX. SPECIFIC CPM
1 32
1 160
1·800
1 4000
1 20,000
1·100,000
1 500,000
DILUTION OF ANTI-CEA

Figure 9

Effects of various quantities of rabbit anti-goat-I^{125} (RAG-I^{125}) on detection of specific anti-CEA binding in the indirect radioimmunoassay. 1×10^6 HCT-8R cells were reacted separately with 50 µl of 1:16 dilutions of normal goat serum or goat anti-CEA for 30 minutes at 37°C. Unbound antibodies were removed by washing. Subsequently, tubes were reacted with various amounts of RAG-I^{125} for 30 minutes at 24°C. Unbound counts were removed by washing and specific counts per minute (cpm) were determined by subtracting the average counts bound to cells reacted with normal goat serum from the average counts obtained from cells reacted with goat anti-CEA. Percent maximum counts were calculated relative to specific counts obtained with 100 µl of RAG-I^{125}.


100
90
80
70
60
50
40
30
20
10
0
% MAX. SPECIFIC CPM
100
80
60
40
20
10
0
RAG-I^{125} (µl.)

II. Cell Surface Biology of Carcinoembryonic Antigen

A. Lateral Redistribution of Carcinoembryonic Antigen at the Surface of Viable Colon Carcinoma Cells

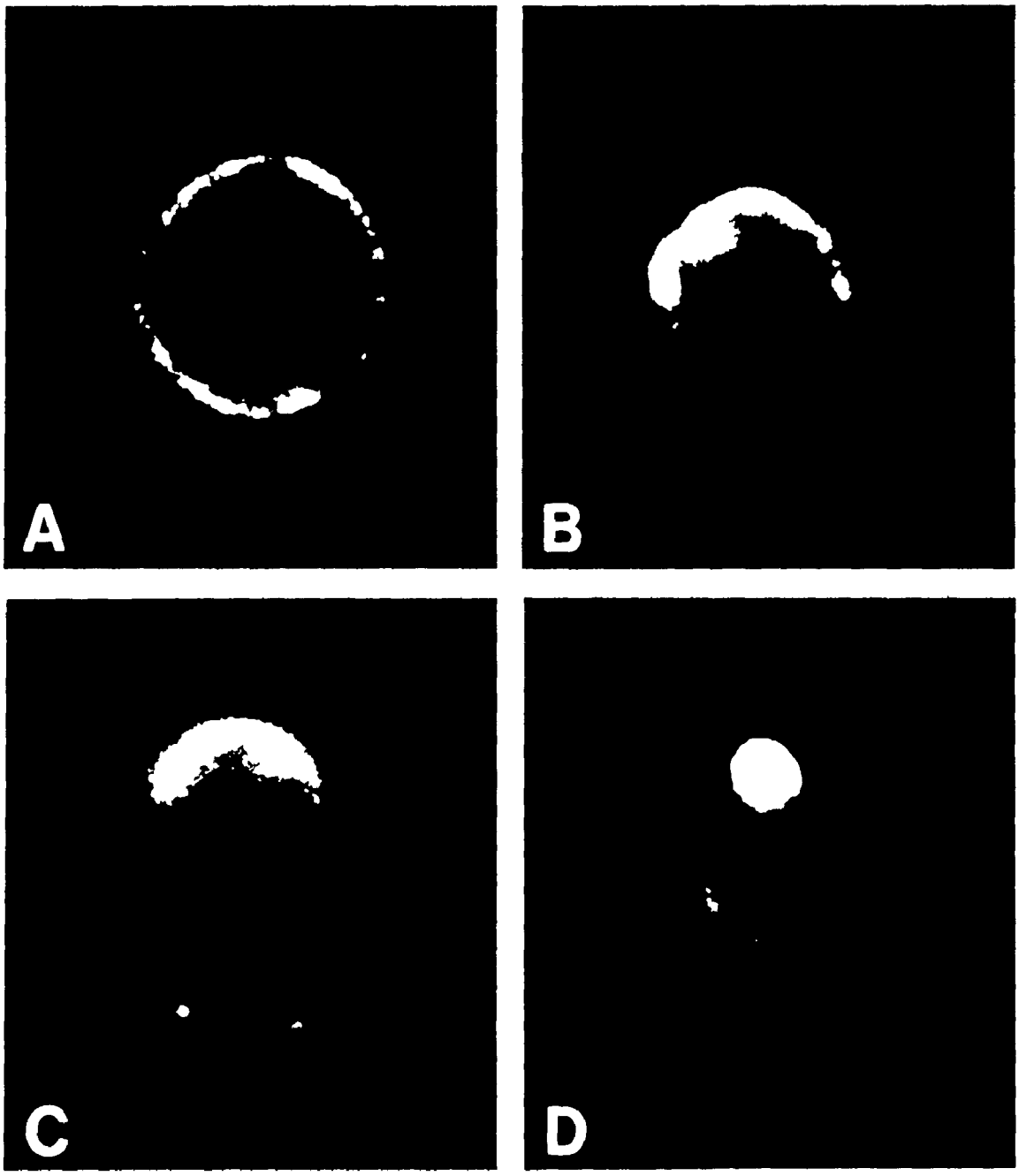
A spectrum of patterns of membrane immunofluorescence were observed subsequent to reacting HCT-8 cells with anti-CEA at 37°C. These ranged from uniform distribution of fluorescence over the cell surface, "ring pattern", to a single crescent of fluorescence at one pole of the cell, "cap pattern" (Figure 10). Enumeration of stained cells according to the distribution of fluorescence revealed a great majority (approximately 75%) of the cells with some degree of eccentric distribution of antigen. This type of immunofluorescence reaction is markedly similar to the phenomenon of antibody-induced capping of certain antigens on the surface of lymphocytes (Taylor *et al.*, 1971; Loor *et al.*, 1972) and suggests that CEA undergoes lateral redistribution on the surface of HCT-8 cells following binding of specific antibodies. It was important to study the dynamics of this process in detail to assure precise measurement of antigen and to possibly shed some light on the biological significance of this surface antigen.

The capping of CEA was studied using both direct and indirect fluorescent (IFA) and radiolabeled antibody thus permitting both visualization and quantitation of antigen;

Figure 10

Indirect immunofluorescence reaction of anti-CEA with HCT-8 cells showing proposed stages of antibody-induced antigen redistribution. Cells were incubated at 37°C with a 1:20 dilution of anti-CEA for various lengths of time. Subsequently, cells were incubated with a 1:10 dilution of fluorescein-conjugated rabbit anti-goat immunoglobulin for 30 minutes at 4°C.

Initial stage of reaction showing antigens evenly distributed over the cell surface giving the ring pattern (A); intermediate stages showing partial redistribution (B and C); and late stage of redistribution showing full cap formation (D). Magnification X 1200.


A
B
C
D

Initial studies were conducted on HCT-8 cells reacted with goat anti-CEA or normal goat serum (NGS) for various lengths of time at 37°C. The cells were then mildly fixed in 2.5% buffered formalin and subsequently reacted with conjugated antiglobulin. These results (Table 3) indicated that initially most (90%) of the stained cells demonstrated the "ring pattern" and within one hour at 37°C the antigen was redistributed on the majority of cells to the "cap pattern". Within 2 to 3 hours, CEA could no longer be detected on the cell surface. A complete lack of fluorescence was obtained with NGS. Thus it appeared that CEA could be induced by specific antibodies to redistribute through a series of steps (Figure 10) until the antigen was selectively removed from the cell surface. The capping reaction appeared to be fairly synchronous with respect to a population of cells as it appeared to begin immediately after addition of antibody (Figure 11).

That the capping was due to the binding of primary antibody was demonstrated by observing the capping sequence both on cells fixed after reacting them with anti-CEA but before the reaction with conjugated anti-globulin and by observing polar redistribution induced in a direct reaction employing fluorescein-conjugated anti-CEA.

Further, the capping of CEA was observed on all CEA-containing cell lines examined (Table 4) and appeared

Table 3

Sequence of Antibody-Induced Redistribution of CEA[a]

Time (hr)	% of Cells[b]			
	Full rings	Half crescent	Polar cap	Not stained
0	90	10	0	0
0.25	85	15	0	0
0.50	75	20	5	0
1.0	40	30	20	10
1.5	10	15	15	50
2.0	5	5	10	80
2.5	0	0	10	90
3.0	0	0	10	90
3.5	0	0	10	90

[a] 5×10^5 HCT-8 cells were reacted with 1:16 dilution of anti-CEA for 30 minutes at 24°C. All tubes were washed and incubated at 37°C for various lengths of time, then subsequently fixed with 2.5% buffered formalin and subsequently reacted with 1:10 dilution of fluorescein conjugated rabbit anti-goat immunoglobulins. Controls which were negative included cells reacted with normal goat serum and conjugate alone.

[b] Percentage of stained cells demonstrating ring, early or late caps and negative fluorescent staining patterns. Results are based on estimates of 3 experiments and involved counts of at least 50 cells/time.

Figure 11

Synchrony of antibody-induced antigen redistribution in a population of HCT-8R cells. 1×10^6 cells were each reacted with 50 µl of a 1:20 dilution of anti-CEA at 37°C for 30 minutes. Unbound antibody was removed by washing followed by reaction with 50 µl of a 1:10 dilution of fluorescein conjugated rabbit anti-goat IgG for 30 minutes at 37°C. Most cells demonstrate late stage of capping. Magnification X 940.

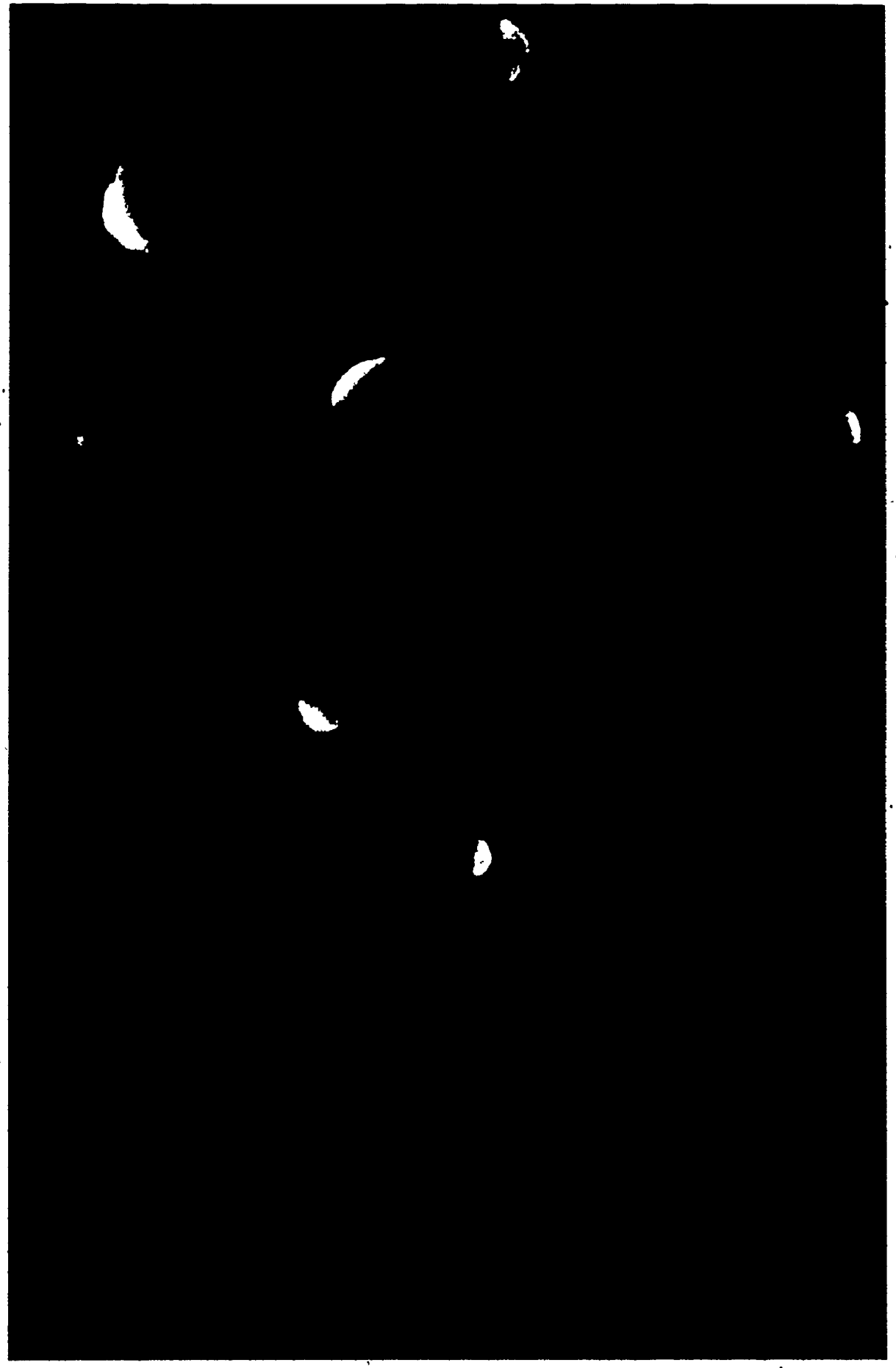


Table 4

Cells Demonstrating Ability to Undergo [redacted]body-Induced Redistribution of CEA[a]

Cell designation	Presence of CEA	Ability to cap
HCT-8	+	+
HCT-8R	+	+
HRT-18	+	+
HT-29	+	+
HEp	-	-
KB	-	-
Hep-2	-	-
HCF	-	-

[a] 1×10^6 trypsinized cells of each strain were incubated with 50 µl of 1:16 dilution of anti-CEA for 30 minutes at 37°C. Unbound counts were removed by washing and subsequently cells were reacted with 50 µl of 1:10 dilution of fluorescein-conjugated rabbit anti-goat immunoglobulin. Cells were then viewed under the ultra-violet microscope for presence of surface CEA and its ability to be redistributed by anti-CEA antibody. Controls consisted of reacting cells with normal goat serum; all these reactions were negative.

to follow a similar pattern and time course in all the cell lines.

B. Factors Affecting the Polar Redistribution of Carcinoembryonic Antigen

The effect of temperature on antibody-induced capping of CEA was examined by performing the IFA at 4°C and 37°C. Reaction of cells with antiserum followed by incubation at 37°C resulted in the typical capping reaction with apparent complete removal of antigen in 2 to 3 hours. In contrast, cells placed at 4°C demonstrated a low percentage (5-10%) of cells showing evidence of capping and most of the cells showed a uniform ring of fluorescence (Table 5). Within 30 minutes of placing these preparations at 37°C, the antigen was observed to undergo capping. Thus CEA capping was found to be temperature-dependent and low temperature inhibition of capping was reversible by increasing the temperature.

The effect of selected chemicals on the antibody-induced capping of CEA was examined (Table 6). Capping was reduced 50 and 20% by 10^{-2} and 10^{-3} M sodium azide, respectively. Sodium azide is an inhibitor of cellular metabolism hence CEA capping appeared to be an energy-dependent phenomenon.

Table 5

Effect of Low Temperature on Antibody-Induced Redistribution of CEA[a]

Antiserum[b]	Time (hr)	Temp. (°C)	Percentage of cells[c]		
			ring	cap	negative
Anti-CEA	0	0	90	10	-
NGS	0	0	-	-	-
Tris	0	0	-	-	-
Anti-CEA	.5	0	90	10	-
Anti-CEA	.5	37	80	20	-
Anti-CEA	1	0	90	10	-
Anti-CEA	1	37	50	50	-
Anti-CEA	1.5	0	90	10	-
Anti-CEA	1.5	37	10	50	40
Anti-CEA	2	0	90	10	-
Anti-CEA	2	37	-	10	90
Anti-CEA	3	0	90	10	-
Anti-CEA	3	37	-	10	90

[a] 1×10^6 HCT-8R cells were reacted with 50 µl of 1:16 dilution of antiserum. Unbound antibodies were removed by washing the cells. Cells were then incubated at either 37°C or 0°C for various lengths of time. Subsequently, cells were reacted with 50 µl of 1:10 dilution of fluorescein conjugated rabbit anti-goat IgG for 30 minutes at 4°C, washed, and observed for percentage of cells demonstrating various antigen distributions.

[b] Cells were reacted with goat anti-CEA, normal goat serum (NGS) or Tris buffer. All cells reacted with NGS or Tris were negative.

[c] Results based on the estimates of three experiments.

Table 6

Effects of Various Inhibitors on Antibody-Induced Redistribution of CEA[a]

Reagent	Concentration	Percent inhibition of capping[b]
NaN_3	10^{-3} M	20
	10^{-2} M	50
Cytochalasin B	1 µg/ml	0
	10 µg/ml	25
Colchicine	10^{-4} M	0
	10^{-3} M	0
Cycloheximide	10 µg/ml	0
	1 mg/ml	0

[a] 1×10^6 HCT-8R cells were pre-incubated for 30 minutes in phosphate buffered saline (PBS) containing indicated concentrations of inhibitors or 2% fetal calf serum as control. Cells were then incubated with 50 µl of 1:20 dilution of anti-CEA for 30 minutes at 24°C. Unbound antibody was removed by washing; cells were then warmed to 37°C, placed in PBS containing inhibitor and examined 0, .5, 1 and 1.5 hours later after reaction with 50 µl of 1:10 dilution of fluorescein conjugated rabbit anti-goat IgG.

[b] Percent inhibition is calculated relative to control which demonstrated 20 caps of 50 cells. Results are average of two experiments.

Capping in numerous systems (Schreiner & Unanue, 1977) has been shown to be dependent on intact submembranous microfilaments and/or microtubules. Consequently, the effects of colchicine and cytochalasin B, disrupters of microtubules and microfilaments, were studied (Table 6). No inhibition of capping was observed when cells were pretreated with colchicine or 1 µg/ml of cytochalasin B but 10 µg/ml of cytochalasin B was seen to inhibit capping by 25%. This suggested that microfilaments may play a role in the capping of CEA.

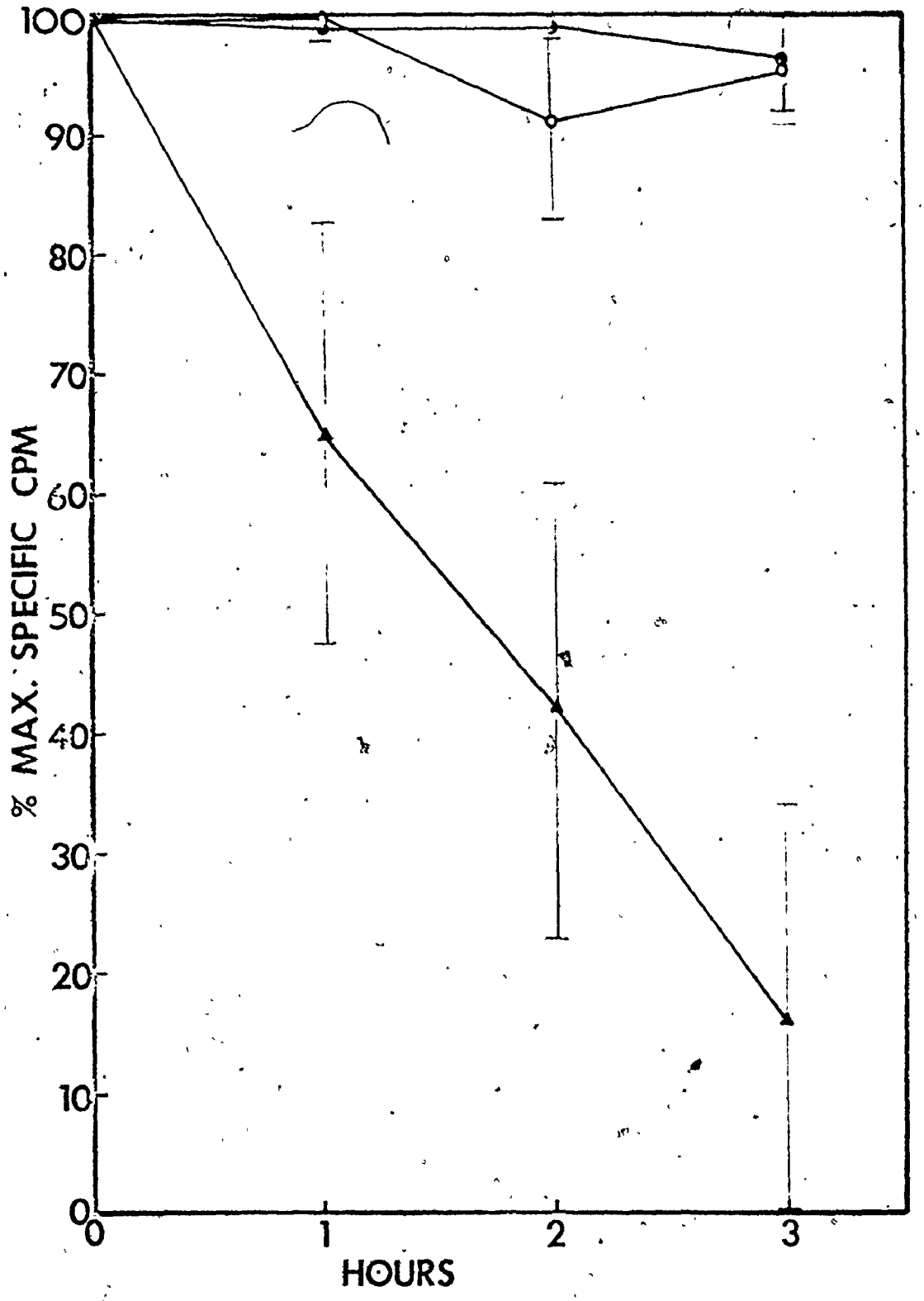
With both sodium azide and cytochalasin B, a patchy network of fluorescence was observed on the cell surface but antigen failed to migrate to one pole of the cell.

No inhibition of capping was observed when cells were pretreated with cycloheximide hence it appeared that no protein synthesis was required for CEA capping (Table 6).

The indirect radioimmunoassay for cell surface CEA was used in conjunction with immunofluorescence techniques to verify and extend observations made using fluorescence alone. These studies supported previous demonstrations that capping was due to the binding of specific anti-CEA (primary) antibodies and that 80% of the antigen was removed from the cell surface in 2-3 hours at 37°C (Figure 12). Less than 10% of the CEA-antibody complexes were removed from the cell surface when incubated

Figure 12

Time required for antibody-induced removal of carcinoembryonic antigen and the effect of low temperature and sodium azide on this process as demonstrated by the indirect radioimmunoassay. 5×10^5 HCT-8R cells were preincubated in Tris containing 10^{-2} M NaN_3 (o) or 2% fetal calf serum (FCS). Cells were then reacted for 30 minutes at 37°C with 50 µl of anti-CEA or normal goat serum (NGS) in duplicate. Unbound antibodies were removed by washing. Cells were then incubated for various lengths of time in buffer containing 10^{-2}M NaN_3 at 37°C (o) or in buffer containing 2% FCS at 0°C (●) or at 37°C (▲). At the end of the period of incubation, each tube was reacted with 50 µl of rabbit anti-goat-I^{125} IgG for 30 minutes at 24°C. Specific counts per minute (cpm) were calculated by subtracting the average of counts bound to cells incubated with NGS from counts bound to cells incubated with anti-CEA. Percent maximum specific cmp were based on counts obtained at time 0. Results presented indicate the mean of four experiments ± one standard deviation.


100
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% MAX. SPECIFIC CPM
0
1
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3
HOURS

at 0°C or in the presence of sodium azide (Figure 12), thus these studies confirmed the temperature and energy dependence of this phenomenon.

It has been shown by two techniques, that in the presence of specific antibodies after 2-3 hours at 37°C, CEA could be selectively removed from the cell surface. Two points remained to be determined; the first was to demonstrate the disposition or fate of the complexes, and the second was to determine whether and when new antigen would reappear at the cell surface.

C. Endocytosis of Carcinoembryonic Antigen-Antibody Complexes

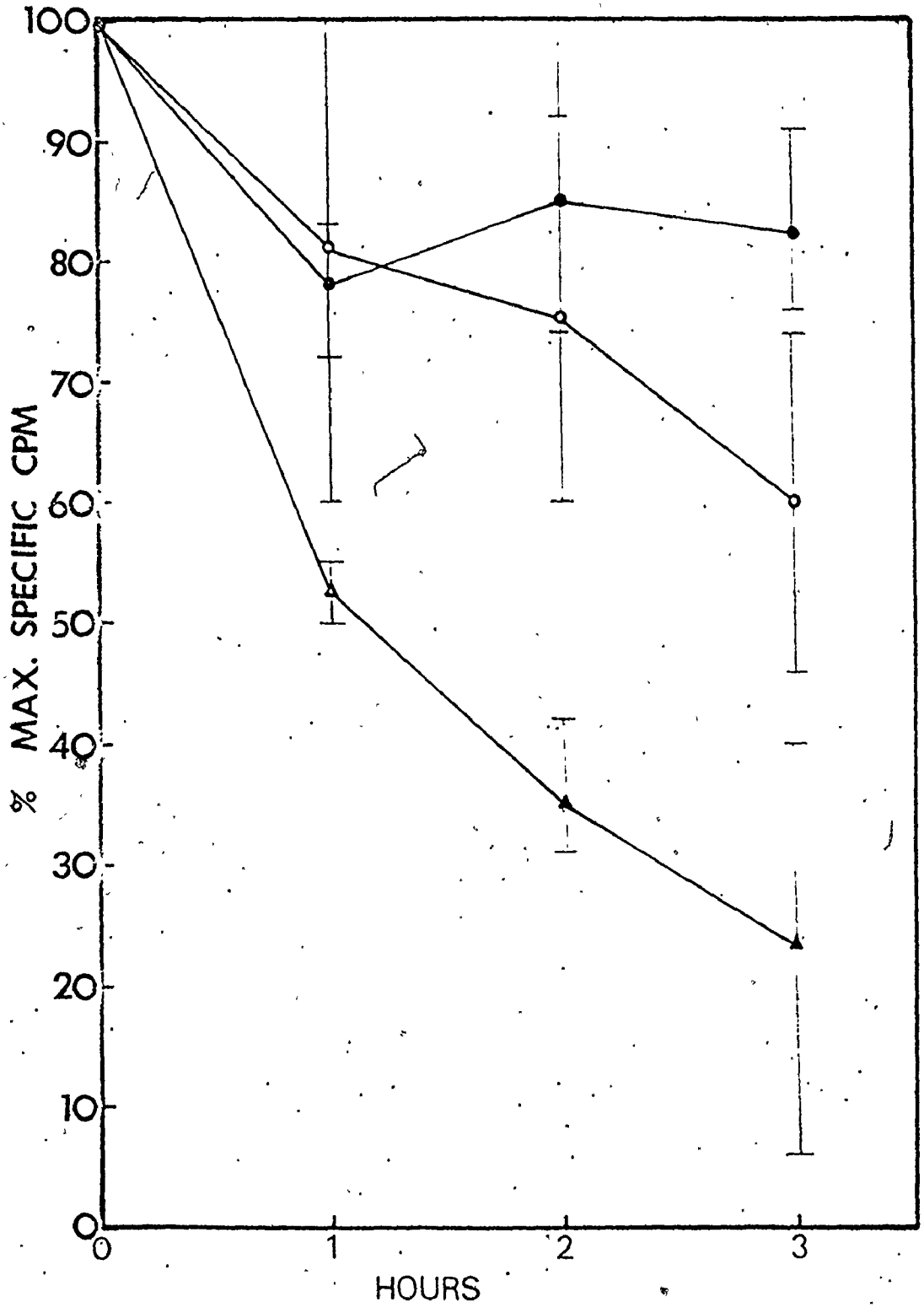
Earlier observations (Rosenthal *et al.*, 1975) suggested that antigen-antibody complexes were shed from the cell surface, as dense fluorescent masses were observed on raised portions of the membrane and fluorescent debris was frequently visible around cells that had lost membrane fluorescence as a result of the capping process. Radiolabeled antibodies proved to be valuable probes for demonstrating that the majority of the complexes were endocytosed after capping. To distinguish between shedding and endocytosis of antigen-antibody complexes, the indirect radioimmunoassay was conducted in two manners. In the first method, unlabeled anti-CEA serum was added to cells which were subsequently incubated at 37°C for various lengths of time

followed by reaction with iodine labeled rabbit anti-goat IgG (RAG-I^{125}). This technique detected complexes on the cell surface only. In the other method, RAG-I^{125} was added immediately after the cells were reacted with anti-CEA serum but before the cells were incubated at 37°C for various time periods. This technique provided a means of detecting antigen-antibody complexes both on the cell surface and within the cell. A loss of the total cell-associated counts over time would suggest the complexes were being shed. Conversely, if these counts remained high at the time that cell surface complexes could no longer be detected, it would indicate the complexes were being endocytosed. As can be seen in Figure 13, while detectable CEA-anti-CEA complexes decreased progressively with time, complexes which had previously bound RAG-I^{125} remained cell-associated. Further, cells reacted with RAG-I^{125} and then placed at 0°C, where neither endocytosis nor shedding could occur, retained almost all radioactive counts. Thus, it appears that the majority of CEA-anti-CEA complexes were endocytosed.

These observations were supported by studies conducted with iodine-labeled anti-CEA, which demonstrated a marginal loss of counts over time while incubating under conditions favoring capping.

Figure 13

Demonstration of antigen-antibody endocytosis by indirect radioimmunoassay. 5×10^6 HCT-8R cells were each incubated with 50 µl of a 1:20 dilution of anti-CEA or normal goat serum (NGS) for 30 minutes at 37°C in duplicate. Unbound antibody was removed by washing. Two groups of tubes were immediately reacted with 50 µl of rabbit-anti-goat-I^{125} IgG (RAG-I^{125}) for 30 minutes at 24°C, washed and placed either at 0°C (●) or at 37°C (○) for various lengths of time. A third group of tubes were incubated at 37°C for various lengths of time before reacting them with RAG-I^{125} (▲). Unbound counts were removed by washing and specific bound counts per minute (cpm) were calculated by subtracting counts bound to cells reacted with NGS from counts bound to cells reacted with anti-CEA. Percent maximum specific cpm were calculated based on counts obtained at time 0. Figure presents mean of 4 experiments ± one standard deviation.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HOURS

D. Turnover of Carcinoembryonic Antigen at the Cell Surface

The next question approached dealt with establishing the time and conditions required for the turnover or reappearance of CEA molecules at the tumor cell surface. These studies first required determination of a concentration of cycloheximide capable of inhibiting protein synthesis in the tumor cells. Figure 14 indicates that 25 µg of cycloheximide/ml maximally inhibited protein synthesis. It was also demonstrated that a thirty minute preincubation with this concentration of cycloheximide did not alter the amount of cell surface CEA.

Next, it was determined that unlabeled goat anti-CEA serum when added to the tumor cells would inhibit the binding of ^{125}I-labeled anti-CEA (Figure 15). The greater the concentration of unlabeled anti-CEA, the greater the inhibition of binding of iodine-labeled anti-CEA (Figure 15). It was assumed that subsequent to the binding of unlabeled anti-CEA, if cells were monitored for increased binding of iodine-labeled anti-CEA then the time and conditions required for the reappearance of CEA could be determined. It was demonstrated (Figure 16) that CEA begins to reappear very rapidly at 37°C and a full complement of CEA was regenerated at the cell surface in about 6 hours. The reappearance of CEA could be totally inhibited by incubation at 0°C and regeneration appeared to require protein

Figure 14

Effects of various concentrations of cycloheximide on protein synthesis of HCT-8R cells. HCT-8R cells were grown to near-confluency in 15 mm petri dishes. Cells were exposed in duplicate to various concentrations of cycloheximide in medium for 15 minutes at 37°C. 0.1-1 μCi/ml of ^{3}H-amino acids were then added to each dish for 1 hour at 37°C. Medium was then removed and cells were washed with phosphate buffered saline (PBS). Cells were subsequently removed with 10 mM EDTA and placed in test tubes. Proteins were then precipitated with 50% TCA for 15 minutes at 4°C. Precipitate was collected on glass fiber filters which were dried and counted in a scintillation counter. Percent maximum ^{3}H-amino acids (% max., ^{3}H-aa) were calculated based on counts obtained from controls incubated with no cycloheximide. Each point represents the average of duplicate determinations.

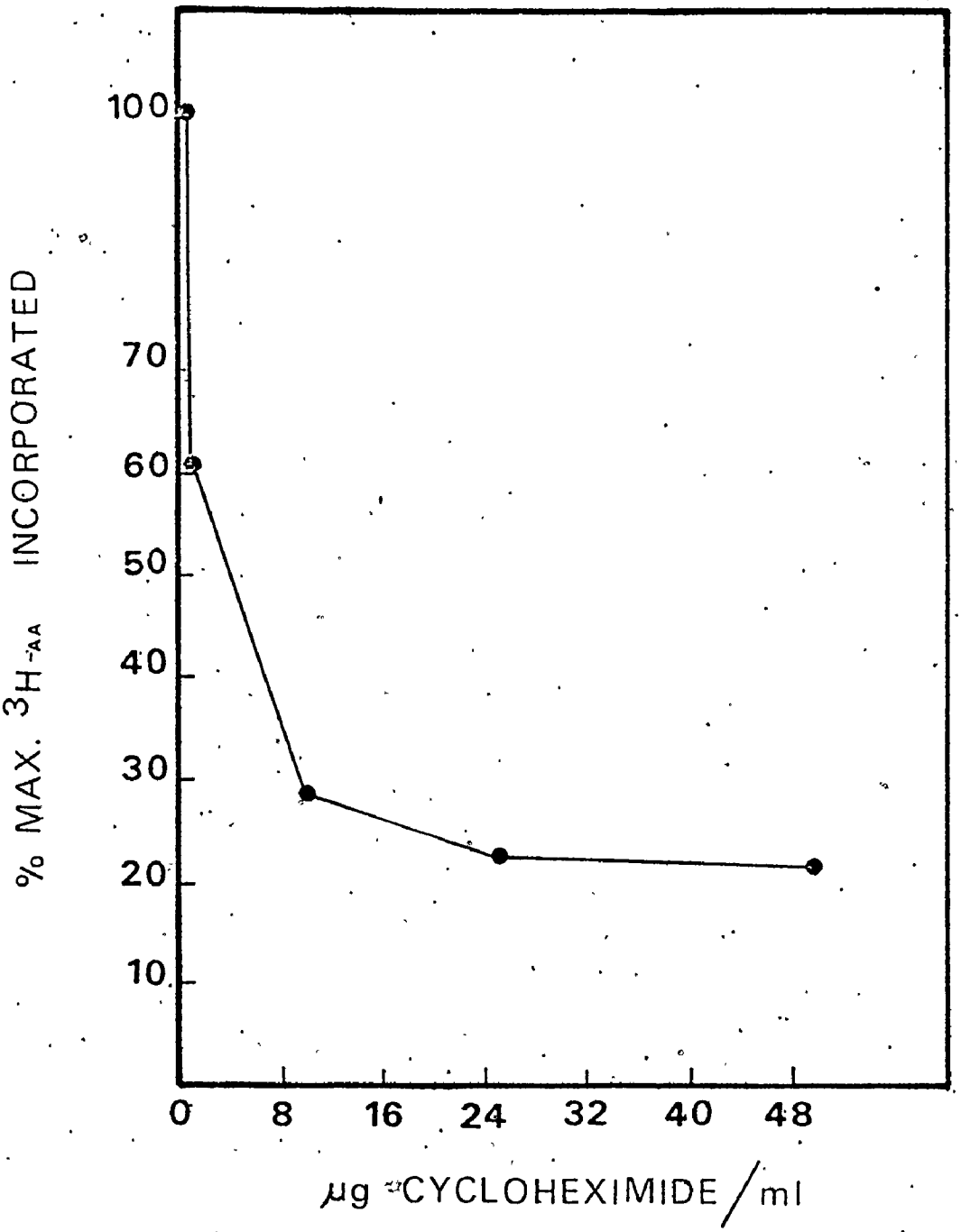
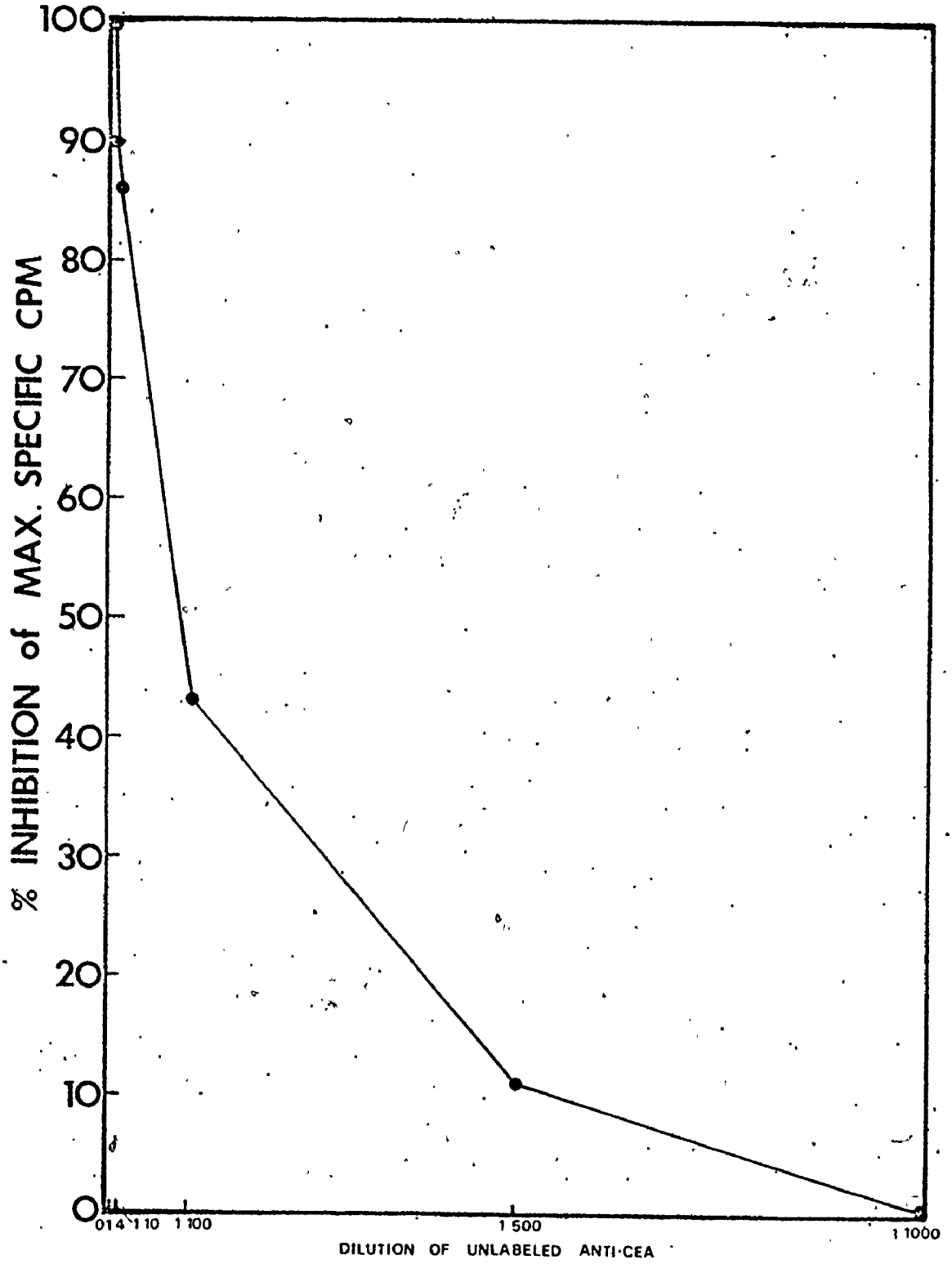

100
70
60
50
40
30
20
10
% MAX. ^{3}H-AA INCORPORATED
0
8
16
24
32
40
48
µg CYCLOHEXIMIDE / ml

Figure 15

Ability of various dilutions of unlabeled anti-CEA to inhibit binding of ^{125}I-anti-CEA IgG. 5×10^6 HCT-8R cells were each incubated with 50 µl of various dilutions of goat anti-CEA for 30 minutes at 24°C. Unbound antibodies were then removed by washing and cells were subsequently reacted with 50 µl of ^{125}I-anti-CEA IgG or ^{125}I-normal goat IgG (^{125}I-NG-IgG) for 45 minutes at 24°C. Unbound counts were removed by washing. Specific counts per minute (cpm) bound to the cells were calculated by subtracting the average CPM obtained with ^{125}I-NG-IgG from cpm obtained with ^{125}I-anti-CEA. Percent maximum inhibition was calculated relative to specific counts obtained in controls run with no unlabeled anti-CEA. Each point represents the average of duplicate determinations.

8 3(a)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10
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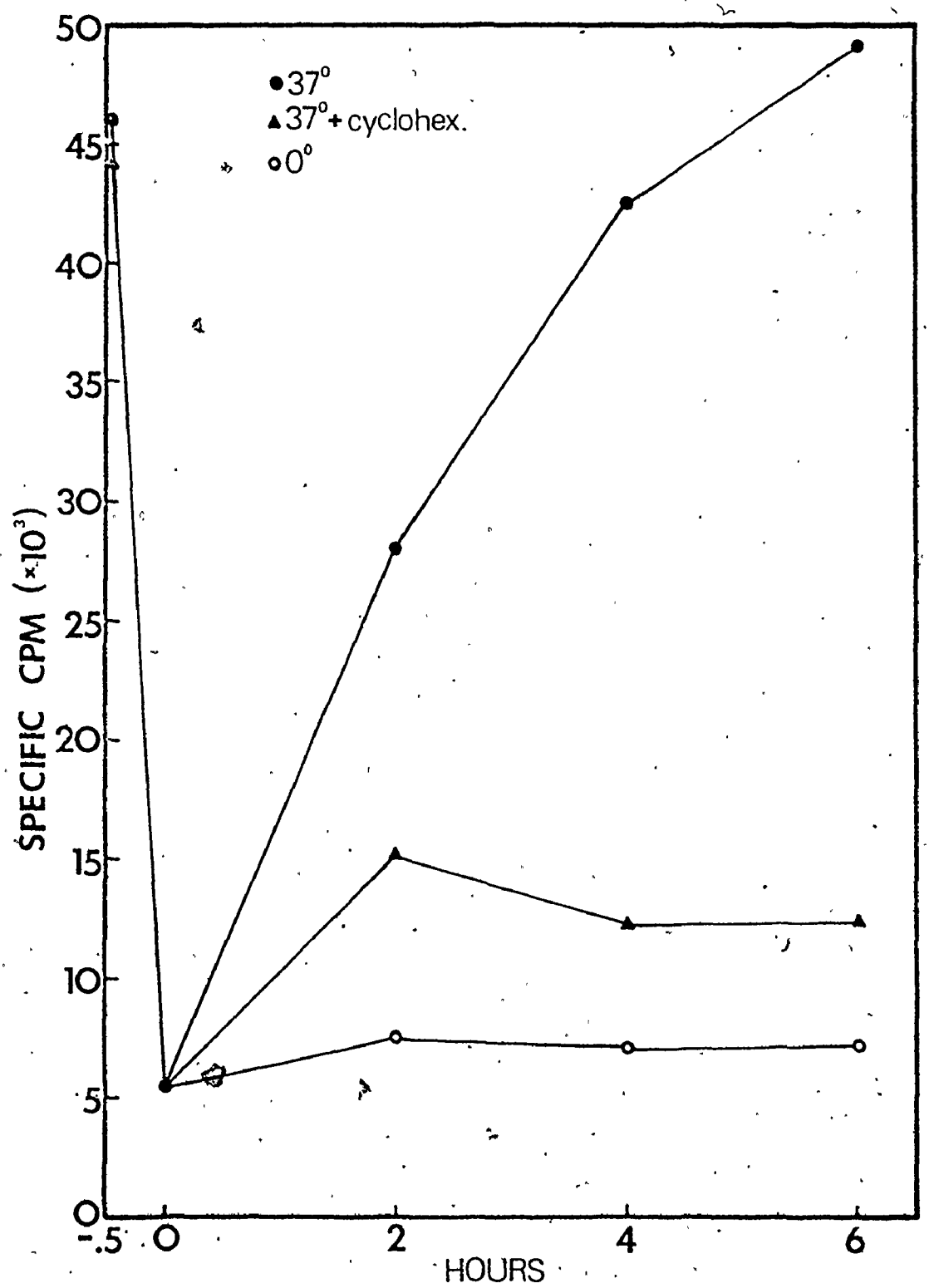
% INHIBITION of MAX. SPECIFIC CPM
0 1 4
1 10
1 100
1 500
1 1000
DILUTION OF UNLABELED ANTI-CEA

Figure 16

Cell surface turnover of carcinoembryonic antigen and requirement for protein synthesis. 5×10^6 HCT-8R cells were each preincubated for 30 minutes at 37°C with either 20 µg/ml of cylcoheximide (▲) or 2% fetal calf serum (● and o). Controls were subsequently incubated with 50 µl of ^{125}I-anti-CEA or ^{125}I-normal goat IgG to obtain the initial maximum specific counts per minute (cpm), while the rest of the tubes received 50 µl of a 1:16 dilution of unlabeled anti-CEA for 30 minutes at 24°C. Unbound antibodies were removed by washing and the cells were then immediately reacted with labeled anti-CEA to establish amount of available cell surface CEA at time zero. The other tubes were placed under three conditions: (●) 37°C in buffer; (o) 0°C in buffer, or (▲) 37°C in buffer containing 20 µg/ml of cycloheximide for various lengths of time. Subsequently, cells were reacted with labeled anti-CEA and normal goat IgG to obtain specific cpm. Specific cpm were calculated by subtracting counts bound to cells reacted with ^{125}I-normal goat IgG from counts obtained on cells reacted with ^{125}I-anti-CEA. Graph depicts a representative summary experiment and each point represents duplicate determinations.


●37°
▲37°+ cyclohex.
○0°
SPECIFIC CPM (×10³)
50
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HOURS

synthesis (Figure 16). Thus, under conditions favoring membrane mobility, capping and protein synthesis, CEA was rapidly regenerated at the cell surface. These results were further verified by indirect immunofluorescence tests which indicated that CEA turnover required about 90 minutes to occur.

E. Comparison of Carcinoembryonic Antigen and Isoantigen A on the Cell Surface

The molecular relationship between CEA and blood group A has been a controversial issue. Some authors have presented evidence that the two antigens represent different determinants on the same molecule (Holburn *et al.*, 1974) or that CEA represents modified or incomplete blood group antigens (Simmons & Perlmann, 1973). Other authors contend that CEA and blood group antigens are distinct molecules (Cooper *et al.*, 1974). The effect of antibody-induced redistribution of CEA afforded a unique opportunity to investigate the relationship between these two molecules. Employing adenocarcinoma cell lines HCT-8, which was derived from a patient with blood group O, and HT-29, which was derived from a blood group A patient, the relationship between isoantigen A and CEA was investigated. The HCT-8 cell line was previously shown (Rosenthal *et al.*, 1977) by the specific red blood cell adherence test to

possess the H blood group antigen but not the A blood group antigen. These results were verified and extended by reacting sera from normal individuals of blood types A, AB, B or O with HCT-8 and HT-29 in the indirect immunofluorescence test. The results shown in Table 7 indicate that all of the sera possessing anti-A antibodies reacted with HT-29 cells, whereas none of the sera reacted with HCT-8 cells. Furthermore, immune adsorbed specific IgG anti-A antibodies reacted with HT-29 but not with HCT-8 cells and this reaction could be abolished by absorption with A type red blood cells. Anti-CEA reacted with and induced capping of CEA on both cell types. In contrast to anti-CEA, anti-A antibodies reacted uniformly on the surface of HT-29 cells giving the ring type reaction and showed no evidence of antigen redistribution when incubated at 37°C for 60 minutes (Figure 17).

Using the fluorescent labels, rhodamine and fluorescein, which produce red and green staining, respectively, the effect of antibody-induced redistribution of CEA on the topography of isoantigen A could be examined on HT-29 cells. The cells were incubated with fluorescein-conjugated anti-CEA for 30 minutes at 37°C to induce capping of CEA. The cells were then washed and mildly fixed with 2.5% buffered formalin and counterstained with IgG or IgM anti-A antibodies which were then reacted with rhodamine-conjugated anti-human immunoglobulin.

Table 7

Reactions of Human Sera Against HCT-8 and HT-29 Cells

Sera subjects with blood type[b]	Surface immunofluorescence[a] reactions using	
	HCT-8	HT-29
A	-	-
B	-	+
AB	-	-
O	-	+
Specific anti-isoantigen A		
Unadsorbed	-	+
Adsorbed with O RBC	-	+
Adsorbed with A RBC	-	-

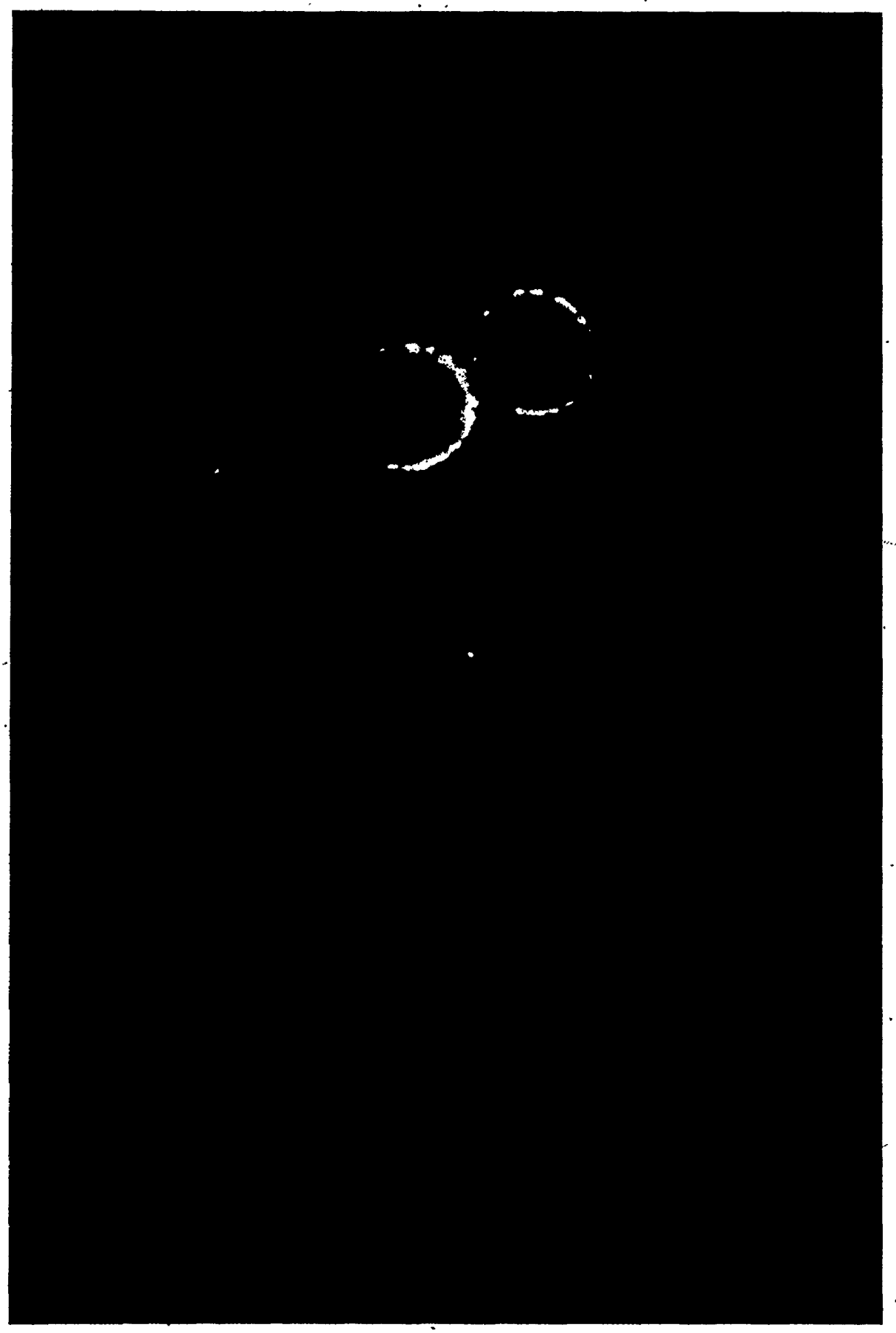
[a] 1×10^5 HCT-8 or HT-29 cells were reacted in the indirect immunofluorescence test with viable cell suspensions and sera obtained from healthy blood donors or specific isoantigen A serum.

[b] Six subjects of each blood type were tested.

Figure 17

Uniform distribution of isoantigen A on the surface of HT-29 cells. 5×10^6 HT-29 cells were incubated with specific anti-A isoantibodies (IgG) for 30 minutes at 37°C. Unbound antibodies were removed by washing and cells were subsequently reacted with a 1:10 dilution of rhodamine-conjugated goat anti-human immunoglobulin for 30 minutes at 37°C. Cells show a ring pattern of immunofluorescence indicating uniform distribution of A antigen on the cell surface. Magnification X 940.

88 (4)



Employing different filter systems in the microscope, the number of cells staining for both isoantigen A and CEA were scored and the location of the two antigens on the surface of each cell was noted.

The results recorded in Table 8 indicate that the polar redistribution of CEA did not appear to alter the distribution of IgG anti-A antibodies on the cell surface. Isoantigen A distributed uniformly over the cell surface with CEA at one pole, was found on 67 out of 80 (84%) cells enumerated. Only 5% of the cells were found to stain for isoantigen A only in the cap which also stained for CEA, while the remaining cells with CEA caps did not stain for isoantigen A. Similar results were obtained when IgM anti-A antibodies were used. Similarly, it was found that binding of anti-A antibodies to HT-29 cells before addition of anti-CEA antibodies did not affect the capping of CEA. Thus, these experiments clearly demonstrate that A antigen and CEA exist as separate molecules on the cell surface and that antibodies to CEA do not cross-react with A antigen. Also, since neither IgG or IgM anti-A antibodies were able to induce capping on HT-29 cells or react with HCT-8 cells it would appear that these antibodies did not react with the CEA molecule which is readily redistributed by specific anti-CEA antibodies.

Table 8

Distribution of Isoantigen A in Relation to Polar Distributed CEA

Pattern of isoantigen A on cells with CEA caps	Experiment 1[a]		Experiment 2[a]		Combined results	
	No.[b] counted	% of total	No.[b] counted	% of total	No. counted	% of total
No A	6	15	3	8	9	11
A in cap only	3	8	1	2	4	5
A distributed over entire cell surface	31	77	36	90	67	84

[a] 5×10^5 HT-29 cells were reacted with fluorescein-conjugated anti-CEA for 30 minutes at 37°C. The cells were washed and fixed with 2.5% buffered formalin for 4 minutes. Cells were then reacted with specific antibodies to isoantigen A for 30 minutes at 4°C, washed and reacted with rhodamine-conjugated goat anti-human globulin.

[b] Counts were made by first identifying a cell with polar distributed CEA. The same cell was then visualized for rhodamine staining by altering the microscope filters and the distribution of isoantigen A recorded.

III. Investigations Concerning the Biological Significance of Carcinoembryonic Antigen

A. Derivation and Characterization of the HCT-8R Substrain

Foci of morphologically altered cells had previously been observed in monolayers of the uncloned parental line, HCT-8, which was derived from a biopsy of a human colon adenocarcinoma (Tompkins *et al.*, 1974). These foci were aspirated from the monolayers, grown in culture and subsequently cloned. The clonal variant was designated HCT-8R (Rosenthal *et al.*, 1977).

Both strains were then characterized (Tompkins *et al.*, 1974; Rosenthal *et al.*, 1977). Morphologically, HCT-8R cells appeared pleomorphic and colonies contained fusiform, epithelial-like and occasional giant cells Figure 18, 1b). This morphology was distinctly different from colonies of HCT-8, which contained tightly packed epithelial cells (Figure 18, 1a). Earlier work (Rosenthal *et al.*, 1977) also established that the two strains differed ultrastructurally, in that HCT-8R cells lacked tight junctions, which were found in HCT-8 cells, and possessed microvilli which were irregular in length, shape and spacing, as opposed to the regular brush-border appearance of microvilli on HCT-8 cells (Tompkins *et al.*, 1974).

Figure 18

Phase-contrast photomicrographs of various human colon carcinoma derived cell strains.

1a HCT-8

1b HCT-8R

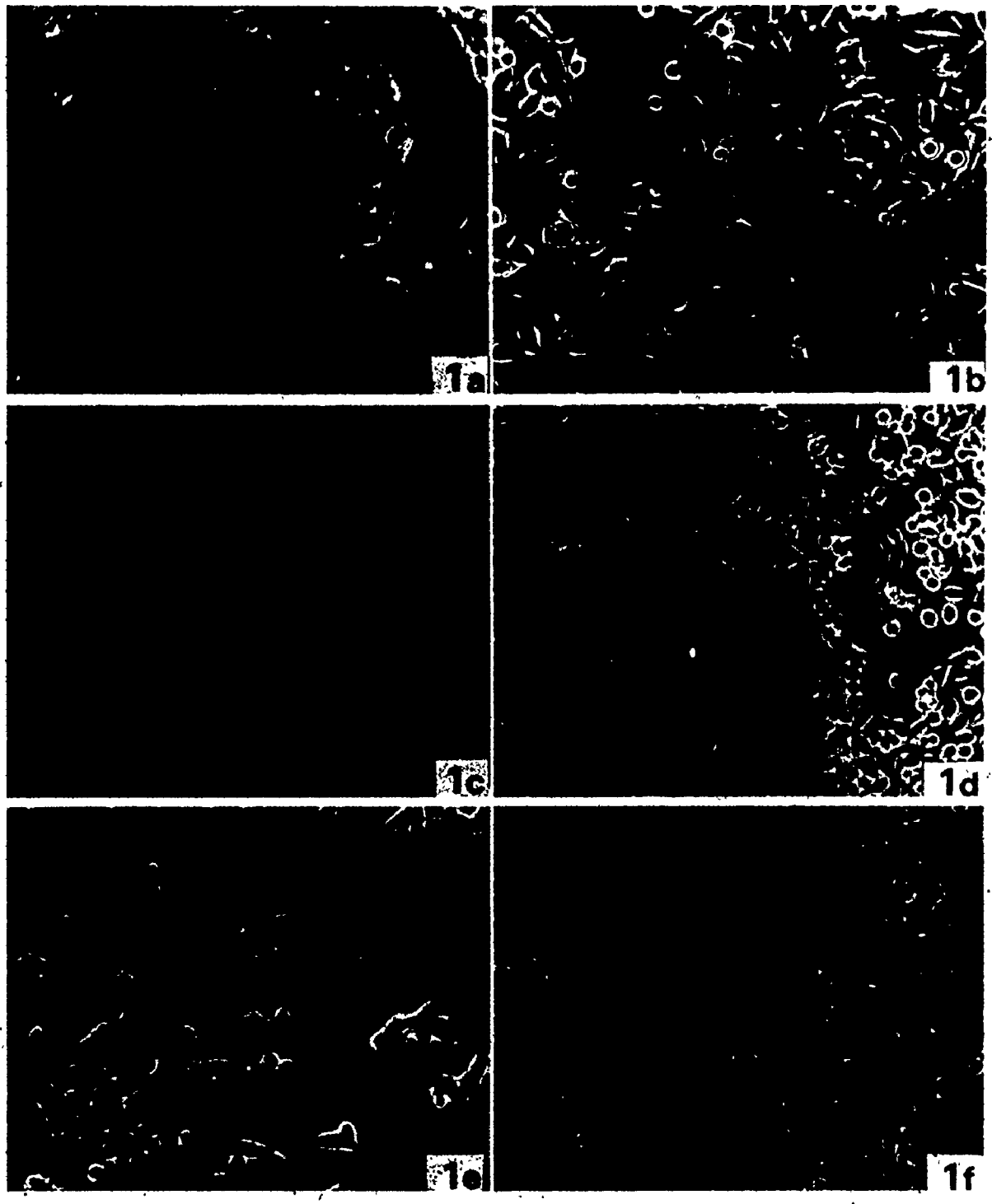
1c HCT-8 Nu1

1d HCT-8R Nu1

1e HCT-8 Nu2

1f HCT-8R Nu2

Magnification X 100.


1a
1b
1c
1d
1e
1f

The *in vitro* growth properties of the two strains were shown to be similar (Rosenthal *et al.*, 1977) and both strains were shown to possess the H blood group antigen (Rosenthal *et al.*, 1977). The cells were shown to differ greatly in their ability to grow in soft agar (Rosenthal *et al.*, 1977). While 75% of HCT-8 cells plated in soft agar formed colonies, no colonies were formed by HCT-8R cells in soft agar.

Earlier cytogenetic studies (Rosenthal *et al.*, 1977) indicated that both strains possessed a modal number of 48 chromosomes. Both strains were shown to possess an X and Y chromosome, thus supporting their male origin (Tompkins *et al.*, 1974).

Initially, the production of CEA by the two strains was examined by indirect immunofluorescence on viable cells. The results presented in Table 9 indicated that while cells of the cloned HCT-8R strain were uniform and all demonstrated bright fluorescence, cells of the uncloned HCT-8 strain were heterogeneous with respect to surface staining. Thirty percent of HCT-8 cells were brightly fluorescent and 40% of the cells appeared negative.

The amount of cell surface CEA was then quantitated with the use of ^{125}I-labeled anti-CEA IgG. The results, Table 9, indicated that the HCT-8R cells possessed almost three times as much cell surface CEA as the HCT-8 cells.

The amount of CEA present in the culture supernatant was also examined by radioimmunoassay (performed by Dr. P. Dent). These results (Table 9) directly correlated with results obtained by the two previous techniques and suggested that the two strains differed markedly in their ability to produce CEA. Further, these results suggest that the quantity of cell surface CEA is directly proportional to the amount of secreted CEA.

The observation of variation of anti-CEA surface staining among cells of the HCT-8 population using the indirect immunofluorescence test could reflect either a heterogeneity among the cells with respect to ability and amount of CEA produced or it could reflect a cell-cycle dependence of CEA production. Since the HCT-8R cells, a clonally derived strain, consistently demonstrated homogeneous bright fluorescence and since three separate techniques indicated a difference in the level of CEA produced, the possibility that the uncloned HCT-8 population was heterogeneous with respect to CEA synthesis seemed more favorable. To clearly demonstrate this, single cell clones from each of the two strains were grown into populations which were subsequently examined by the direct radioimmunoassay to quantitate the amount of cell surface CEA. Results presented in Figure 19 indicated that clones derived from the HCT-8R population demonstrated a normal distribution at a higher level of CEA production relative to the HCT-8

Table 9

CEA Production by Various Cell Strains Derived from a Human Colon Carcinoma

Cell strain	Immunofluorescence % of cells staining[a]			Binding of ^{125}I-labeled anti-CEA			CEA in culture media (ng/ml[b])
	++	+	-	cpm[c]	ratio[d]		
HEI		ND[e]		2,565	1.0		0.0
HCT-8	30	30	40	8,129	4.7	1.6	3.3
HCT-8 Nu1	50	0	50	9,207	4.2	0.6	5.1
HCT-8 Nu2	5	0	95	5,644	2.3	0.3	0.1
HCT-8R	100	0	0	28,102	10.7	1.3	23.2
HCT-8R Nu1	50	50	0	11,313	4.3	1.0	18.0
HCT-8R Nu2	100	0	0	15,721	7.3	1.5	>25.0

[a] Percentage of cells staining according to degree of intensity of fluorescence, ++, bright; +, moderate intensity; -, no fluorescence.

[b] CEA concentration in culture medium determined by radioimmunoassay (LeGerfo *et al.*, 1971).

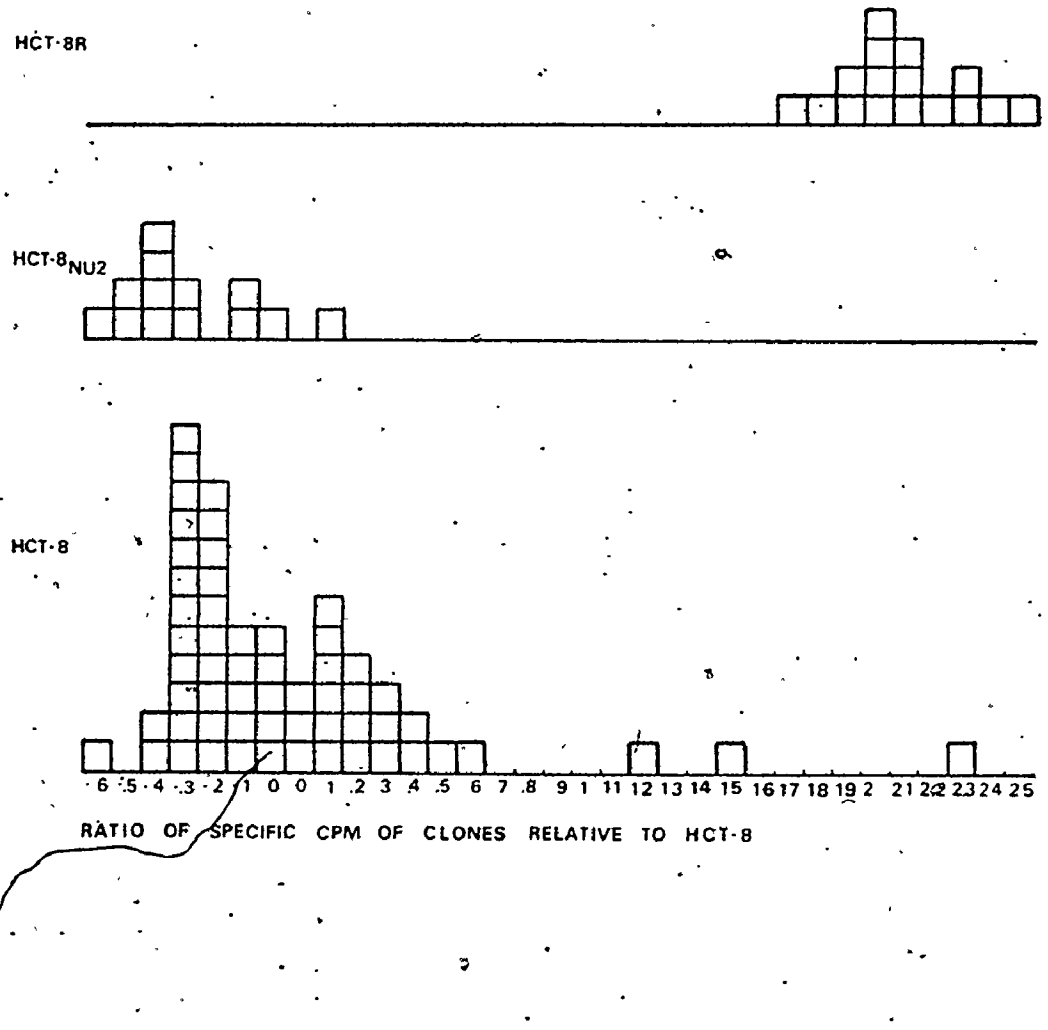
[c] cpm of ^{125}I-labeled anti-CEA IgG specifically bound to 5×10^6 cells; results of a representative experiment shown.

[d] Average ratios of binding ± range derived from 3 experiments. The ratio was derived by dividing cpm for test cell by cpm bound to human embryonic intestinal cells.

[e] ND, not done.

Figure 19

Frequency distribution of CEA expressed by clones derived from various cell strains relative to HCT-8. Single cells of HCT-8, HCT-8R and HCT-8 Nu2 were cloned in microtiter wells and grown into populations. 5×10^6 cells of each clone and of HCT-8 cells (standard) were separately examined for quantity of cell surface CEA employing the direct RIA. 50 µl of ^{125}I-anti-CEA or ^{125}I-normal goat IgG were reacted with the cells for 45 minutes at 24°C. Unbound counts were removed by washing. Specific counts per minute (cpm) were calculated by subtracting counts bound to cells reacted with ^{125}I-normal goat IgG from counts obtained with ^{125}I-anti-CEA. The ratio of specific cpm was calculated by dividing specific cpm of any clone by specific cpm of the standard and subtracting 1 from this number. Each box represents one clone and each clone was tested in duplicate.


HCT-8R
HCT-8NU2
HCT-8
.6 .5 .4 .3 .2 .1 0 0 1 .2 3 4 .5 6 7 .8 9 1 11 12 13 14 15 16 17 18 19 2 21 22 23 24 25
RATIO OF SPECIFIC CPM OF CLONES RELATIVE TO HCT-8

population. Thus, all the cells composing the HCT-8R population appear to express approximately three times as much CEA as the HCT-8 population. This is contrasted by the non-random distribution of CEA production demonstrated by clones derived from the HCT-8 population. These results indicate that cells of the HCT-8 population are heterogeneous with respect to CEA synthesis. These differences, also, could not be attributed to differences in cell size.

Heterogeneity of the HCT-8 population was also supported by the observation that clones derived from HCT-8R resembled HCT-8R morphologically, while clones derived from the HCT-8 population varied in morphology. Further, since 2 of the 58 HCT-8 derived clones expressed levels of cell surface CEA comparable to HCT-8R (Figure 19), it would appear likely that about 5% of the HCT-8 population consisted of HCT-8R cells.

B. Oncogenicity of HCT-8 and HCT-8R in Nude Mice

Having demonstrated that the two strains differed markedly in their abilities to produce CEA, various doses of viable tumor cells were injected subcutaneously on the back of nude mice to determine whether there was an association between elevated CEA production and tumorigenicity. Within 10 days after injection, tumors were visible on mice receiving 10^7 and 10^6 cells of both strains (Table 10).

Table 10

Formation of Tumors in Nude Mice by Injected Human Colon Carcinoma Cells

	No. of mice with tumors after 60 days/no. of mice given injections of cells			
	10^7 cells[a]	10^6 cells	10^5 cells	10^4 cells
HCT-8	5/5	5/5	0/5	0/5
HCT-8R	5/5	4/5	0/5	0/5
HCT-8 Nul	ND[b]	5/5	5/5	2/5
NCT-8R Nul	ND	5/5	4/5	0/5

[a] Viable cells injected subcutaneously on the back of nude mice in 0.1 ml of TBS.

[b] ND, not done.

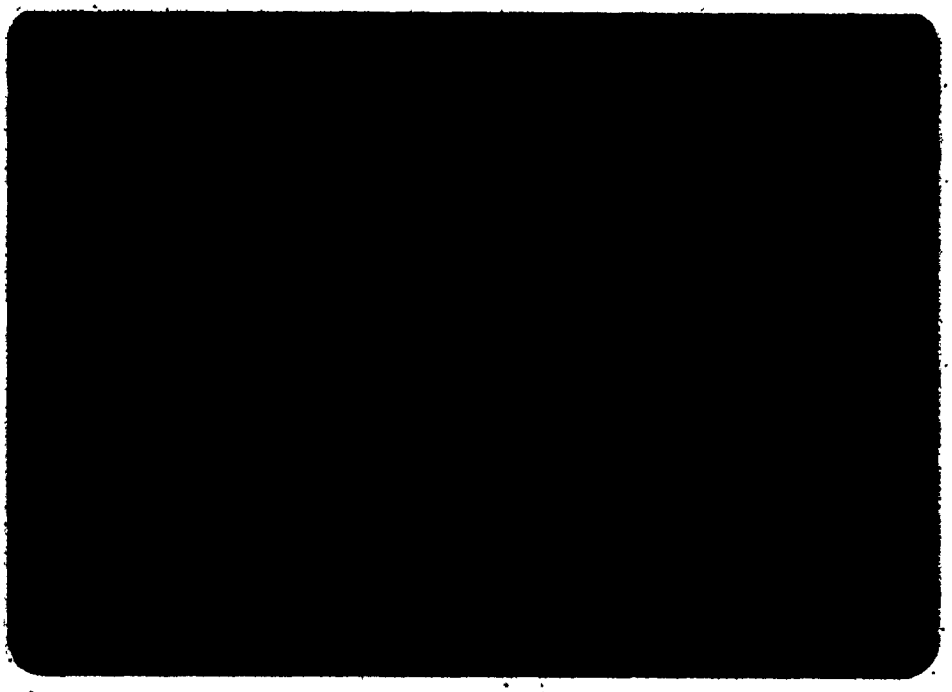
Tumors grew locally and metastasis was never observed. Animals receiving lower tumor cell inoculum were observed for a period of two months and no tumors appeared. Histological examination of tumors formed by HCT-8 cells showed areas of well-differentiated and areas of poorly differentiated adenocarcinoma (Figure 20, top). Both well and poorly differentiated areas of the tumor produced mucin. HCT-8R tumors appeared homogeneously poorly differentiated (Figure 20, bottom) but also produced mucin. The morphological characteristics of tumors formed by both strains were consistent with those found in colonic adenocarcinoma.

An HCT-8 tumor and an HCT-8R tumor were excised from nude mice and established in culture. These strains were designated HCT-8 Nul and HCT-8R Nul, respectively. The HCT-8R Nul cells maintained the HCT-8R morphology, but the cells were smaller (Figure 18, 1d). Measurements of trypsinized cells indicated that while the majority of HCT-8R cells had diameters of 3.5 to 4 μM, the HCT-8R Nul cells presented with a very homogeneous cell size of 2.5 μM in diameter. The HCT-8 Nul cells maintained the morphology and size of the parent HCT-8 cells (Figure 18, 1c).

Following a short period of *in vitro* culture, the nude mouse passaged strains were then reinjected into nude mice. The HCT-8 Nul and HCT-8R Nul cells were able to form tumors in nude mice at lower cell concentrations

Figure 20

Histology of HCT-8-induced tumor in the nude mouse (TOP). Tumor demonstrates area of well and poor differentiation. Histology of HCT-8R-induced tumor in the nude mouse. Tumor demonstrates poor differentiation throughout (BOTTOM). Magnification of both X 100.



(Table 10) than the parent cells. Thus, it appeared that selection of more tumorigenic cells occurred as a result of passage through the nude mice.

Selected tumors formed by HCT-8 Nu1 or HCT-8R Nu1 were established in culture, and these cell strains were designated HCT-8 Nu2 and HCT-8R Nu2. Again the HCT-8R Nu2 cells maintained the morphology and small size of HCT-8R Nu1 cells (Figure 18, 1f). But the HCT-8 Nu2 cells did not maintain the morphology of HCT-8 cells; instead of tightly packed epithelial cells these cells were more fibroblast-like and did not grow in tight colonies (Figure 18, 1e).

Thus, a number of cell lineages were established and characterized with respect to their ability to produce CEA by the three techniques previously mentioned (Table 9). Results obtained by the three techniques correlated well with one another and indicated that while strains derived from the cloned HCT-8R strain all maintained a relatively high level of CEA production, cells derived by passage of HCT-8 cell through nude mice resulted in a strain, HCT-8 Nu2, that produced a very low level of CEA.

Clones of HCT-8 Nu2 were isolated, grown into populations and compared to HCT-8 with respect to their amounts of cell surface CEA. Although only a few HCT-8 Nu2 clones were examined, they appeared to present a normal distribution (Figure 19) and on the average appeared to

produce about half the amount of CEA expressed by HCT-8 cells. Further, about 50% of clones of the HCT-8 population produce levels of CEA comparable to HCT-8 Nu2. This suggests that most of the HCT-8 population consists of low CEA expressing cells, and this belief is supported by fluorescence studies which indicated that about 40% of HCT-8 cells were negative (Table 9).

Thus, strains established from the clonally derived HCT-8R cells were homogeneous with respect to morphology, state of differentiation and CEA production, while strains derived from the uncloned HCT-8 strain differed in these characteristics. Yet, in nude mice, both strains were equally tumorigenic and this property did not appear to reflect the level of CEA synthesis. Hence it appears, at least in nude mice, that CEA does not correlate with tumorigenicity.

C. Cytogenic Analysis of Various Cell Strains

Previous karyotypic analysis was reevaluated with more precise banding techniques on HCT-8, HCT-8R and their respective nude mouse passaged substrains.

The modal number of chromosomes was fairly sharp at 48 for all strains. All cultures from the cloned line (HCT-8R, 8R Nu1 and 8R Nu2) had a distinctive cytogenetic marker designated 7q+ which was found in nearly 100% of all

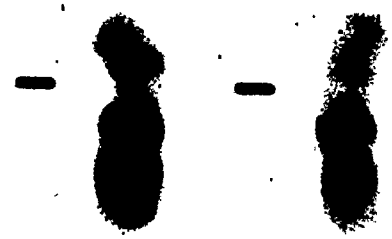
the metaphases examined. The 7q+ marker is a normal human No. 7 chromosome with a chromatid addition at the distal end of the long arms, which has dull fluorescence (Figure 21).

The parent lines HCT-8, HCT-8 Nul and HCT-8 Nu2 had a more heterogeneous pattern, but the majority of the metaphases contained a marker which was designated 4q+ since it has the appearance of a normal No. 4 with a brightly fluorescent chromatid addition on the distal end of the long arms (Figure 21). Occasional examples of the 7q+ were seen particularly in line HCT-8 Nul. The cultures designated HCT-8 Nul and HCT-8 Nu2 also contained a marker Y chromosome in more than 50% of the metaphases. This is formed by the addition of a moderately bright, banded chromatid added to the p or upper arm of an apparently normal Y chromosome (Figure 21). No mouse chromosomes were found in any metaphases.

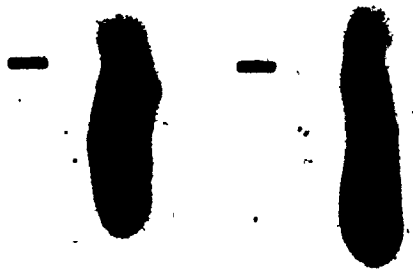
Thus, again, with regard to karyotype, the clonally derived HCT-8R strain and its derivatives appeared homogeneous with respect to marker chromosomes while the HCT-8 strain derivatives demonstrated a more heterogeneous pattern.

Figure 21

Predominant marker chromosomes from various human colon carcinoma-derived cell strains compared with the normal chromosomes. (See text.)



7 7q+



4 4q+

Y marker Y

IV. Factors Controlling the Surface Expression of Carcinoembryonic Antigen

A. Screening of Possible Inducing Agents

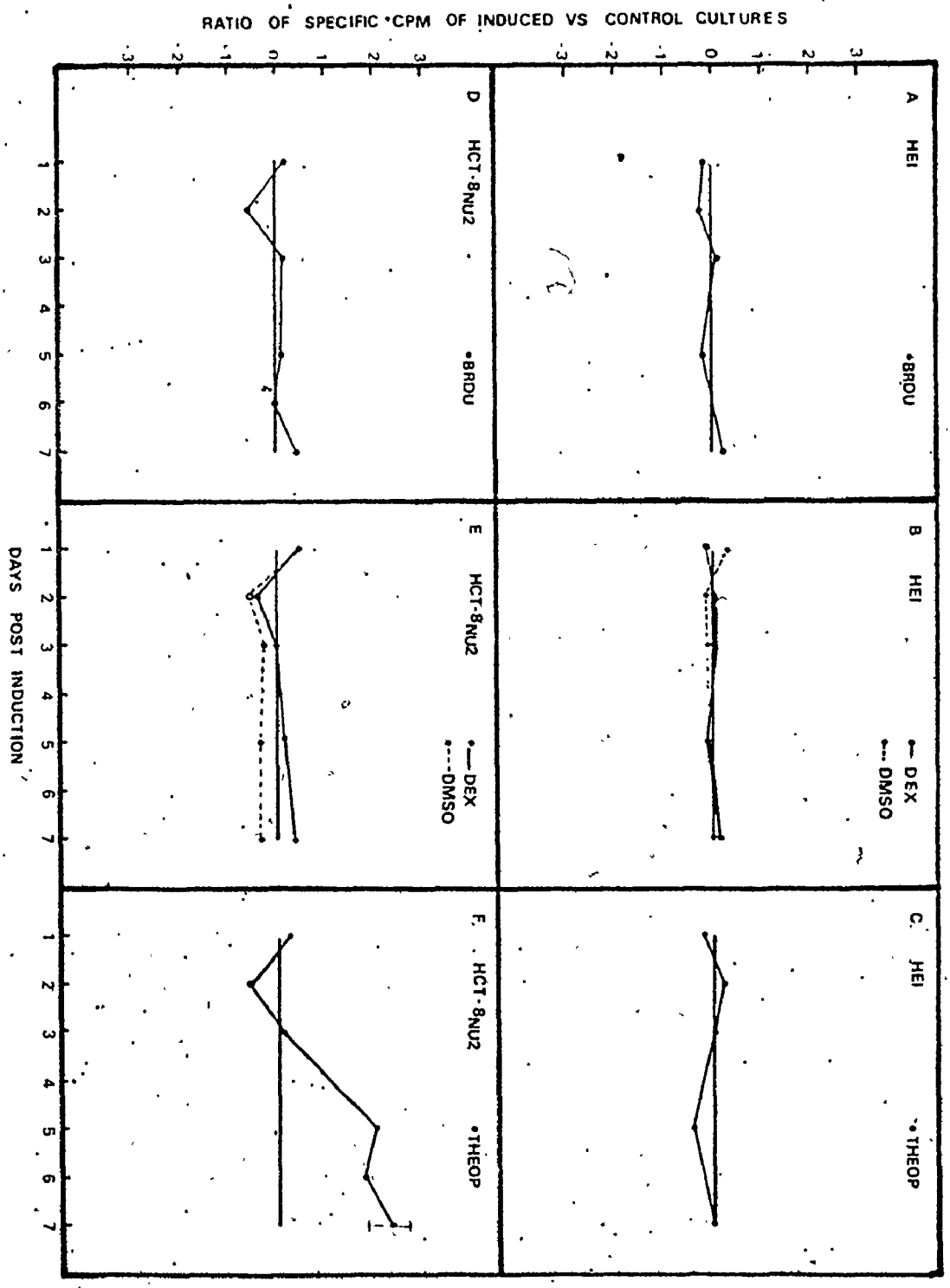
The various cell strains (Table 9) have been in culture now for over two years and all the strains have maintained their relative level of CEA synthesis over this time. This coupled with the previous results demonstrating that the clonally derived HCT-8R strain maintained its high level of CEA synthesis whether subjected to selection in the nude mouse or artificial selection by cloning, suggests that the level of CEA expressed by a cell is heritable, stable and hence tightly controlled.

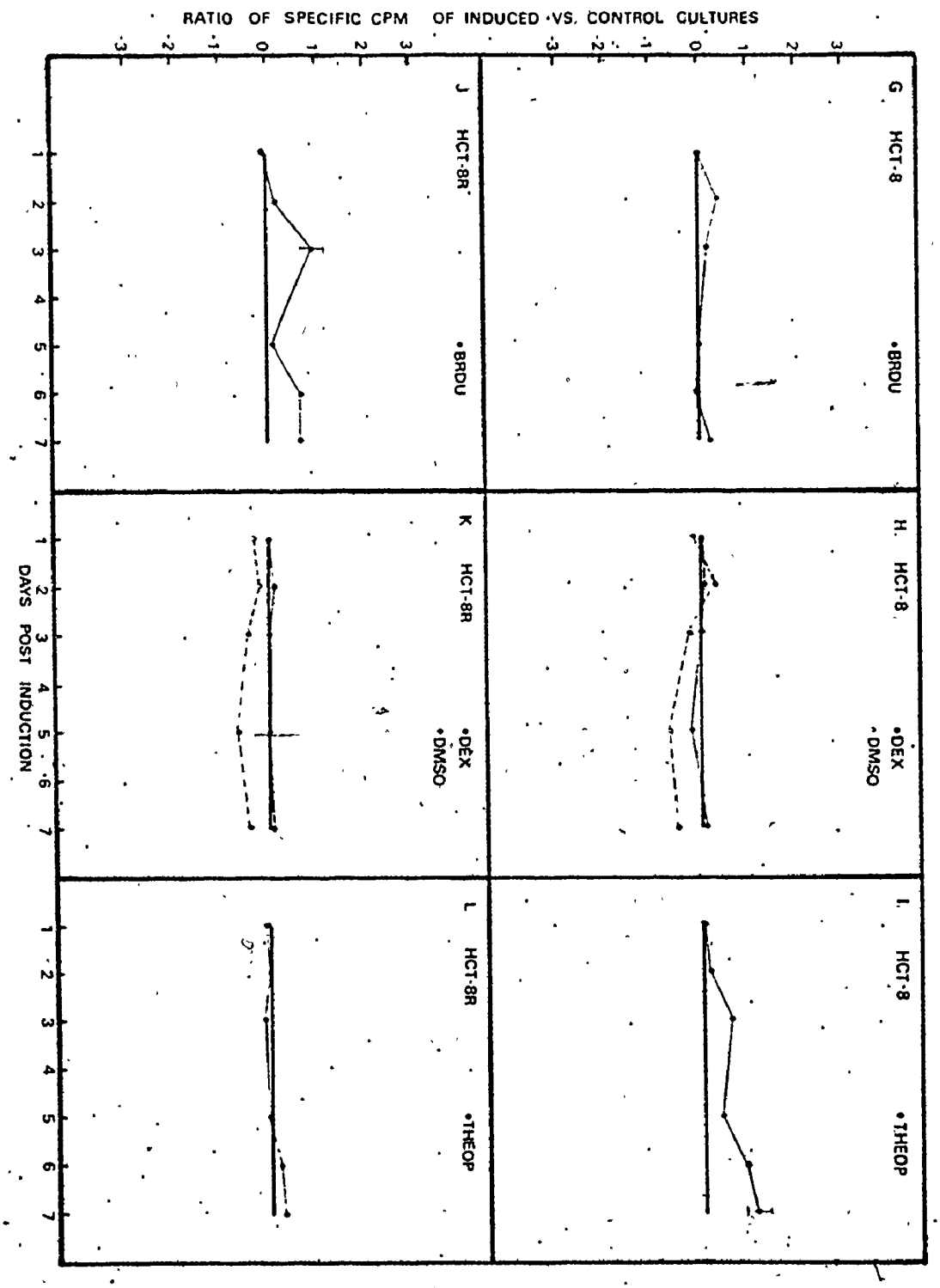
CEA represents an oncodevelopmental glycoprotein as it is found both in the developing fetal gut and in tumor cells. A number of chemical agents known to alter the expression of various "differentiation-associated" products in other experimental systems were examined with respect to their effect on CEA synthesis by the various colon carcinoma cell strains (See Appendix II; Figure 22 A-L). The chemical agents or inducers examined were dimethyl-sulphoxide (DMSO), bromodeoxyuridine (BrdU), dexamethazone, theophylline and dibutyrl cyclic adenosine monophosphate (dbcAMP). Their effects on the non-CEA producing HEI line, the low producing HCT-8 Nu2 strain, the HCT-8 and high

Figure 22 (A-L)

Plots of mean ratios of CEA induction by various inducing agents on various cell strains over time.

For experimental procedure and calculation of results, see Appendix II.


RATIO OF SPECIFIC *CPM OF INDUCED VS CONTROL CULTURES
A HEI *BRDU
B HEI — DEX ---DMSO
C HEI *THEOP
D HCT-8NU2 *BRDU
E HCT-8NU2 — DEX ---DMSO
F HCT-8NU2 *THEOP
DAYS POST INDUCTION


RATIO OF SPECIFIC CPM OF INDUCED VS. CONTROL CULTURES
DAYS POST INDUCTION
G HCT-8 •BRDU
H HCT-8 •DEX •DMSO
I HCT-8 •THEOP
J HCT-8R •BRDU
K HCT-8R •DEX •DMSO
L HCT-8R •THEOP

producing HCT-8R strain were monitored over a seven day period.

As can be seen in Figure 22 F, an increase of CEA synthesis was produced in HCT-8 Nu2 cells by incubation with medium containing 1 mM of theophylline. This effect appeared after day 3 and reached a maximum at day 5 which was maintained through day 7. Similar enhancement of CEA synthesis was observed in the parental HCT-8 cells incubated with theophylline (Figure 22 I) although the increase was not as marked as in HCT-8 Nu2 cultures. This effect suggests that a subpopulation of the HCT-8 strain consists of theophylline-inducible cells and further supports the heterogeneous nature of the HCT-8 population.

Theophylline did not effect the synthesis of CEA by HCT-8R cells, but 3 µg/ml of BrdU was observed to significantly increase the surface expression of CEA transiently on day 3 (Figure 22 J). This enhanced expression could not be detected by day 5. None of the other inducing agents examined affected the synthesis of CEA in these cell strains and no agent was capable of inducing non-CEA producing HEI cells to synthesize CEA.

Growth curves obtained by viable cell counts in trypan blue indicated that no agent, except DMSO (2%), inhibited cell growth; so it appears unlikely that the observations could be attributed to toxic reactions. Also, measurements of individual induced and control cells

indicated that increases in cell surface CEA could not be attributed to differences in cell size.

B. Effects of Different Inducer Concentrations on Carcinoembryonic Antigen Synthesis

After detecting the inducing ability of theophylline on HCT-8 Nu2 and BrdU on HCT-8R cells, dose-response experiments were performed. As can be seen in Figure 23, increasing amounts of theophylline produced increasing synthesis of CEA with a maximum production occurring with 1.5 mM of theophylline. The enhanced level of CEA produced by these cells approaches the level normally observed in non-induced HCT-8R cells.

A similar direct relationship was observed between increasing concentrations of BrdU and CEA synthesis by HCT-8R cells, with maximum expression occurring with 3 µg of BrdU per ml of medium (Figure 24). Further, these results (Table 11) indicated that a 12 hour pulse of BrdU was sufficient to induce CEA synthesis to a level attained by continued exposure of HCT-8R cells to the drug.

C. Lack of Density-Dependence of HCT-8 Nu2 Cells for Induction by Theophylline

CEA induction by theophylline in HCT-8 Nu2 required

Figure 23

Dose-response curve of theophylline on HCT-8 Nu2 cells. Cultures were seeded on day zero with 1-2 x 10^7 cells and allowed 24 hours to enter log phase. On day one, medium was removed from the cells and replaced with medium containing none or different concentrations of theophylline. This medium remained on the cells until day six at which time the cells were examined for cell surface CEA by the direct RIA. Specific counts per minute (cpm) were calculated by subtracting the average cpm bound to 5 x 10^6 cells with ^{125}I-normal goat IgG from average cpm bound with ^{125}I-anti-CEA. Each point represents the average of duplicate determinations.

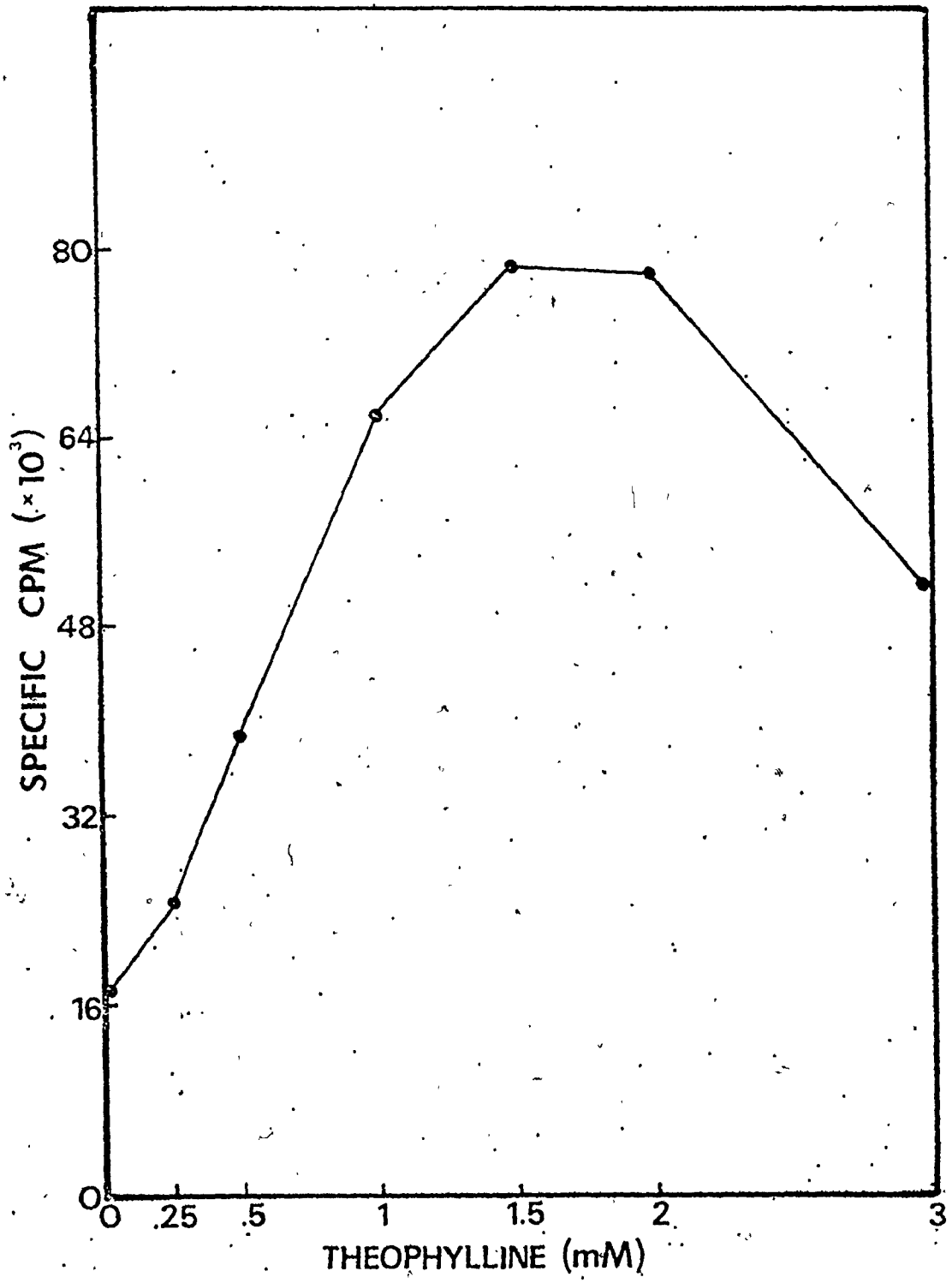

80
64
48
32
16
0
SPECIFIC CPM (×10³)
0
.25
.5
1
1.5
2
3
THEOPHYLLINE (mM)

Figure 24.

Dose-response curve of BrdU on HCT-8R cells. Cultures were seeded with 1×10^7 cells on day zero and allowed 24 hours to enter log phase. On day one, medium was removed and replaced with medium containing none or various concentrations of BrdU. All cultures were wrapped in foil to block out exposure to light. On day 3, cells were examined for cell surface CEA by direct RIA. Specific counts per minute (cpm) were calculated by subtracting counts bound to 5×10^6 cells with ^{125}I-normal goat IgG from counts bound with ^{125}I-anti-CEA. Each point represents the average of duplicate determinations.

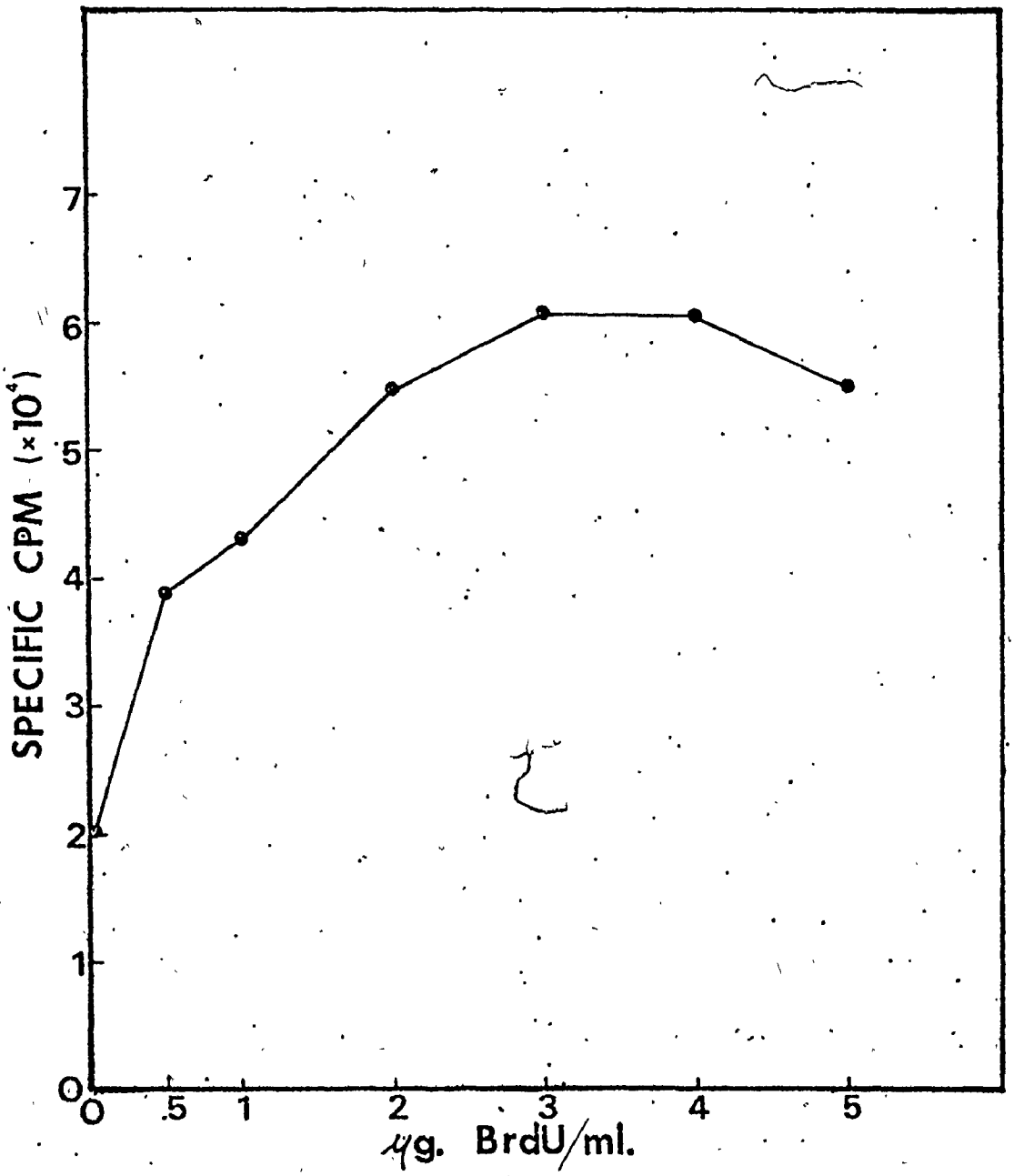

SPECIFIC CPM (×10⁴)
0
1
2
3
4
5
6
7
0
.5
1
2
3
4
5
μg. BrdU/ml.

Table 11

Effects of Different Concentrations of BrdU on CEA Synthesis by HCT-8R Cells[a]

Inducer	Time (hr)[b]	Specific cpm[c]	
		Experiment #1	Experiment #2
None	24	7,556	20,595
BrdU (.5 µg/ml)	24	ND	39,001
BrdU (1 µg/ml)	24	17,270	43,165
BrdU (2 µg/ml)	24	20,563	54,974
BrdU (3 µg/ml)	6	ND	53,516
	12	ND	57,299
	18	ND	56,022
	24	23,313	60,835
BrdU (4 µg/ml)	24	ND	60,665
BrdU (5 µg/ml)	24	ND	55,124

[a] Cultures seeded with 1.5×10^7 cells on day zero, allowed 24 hours to enter log phase at which point inducer is added. NOTE: All cultures were kept in aluminum foil and not exposed to light.

[b] Time indicates the number of hours cells were exposed to medium containing noted concentration of inducer. After this period, cultures were refed with fresh non-inducer-containing medium and cultured for an additional 24 hours before being listed.

[c] (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG = specific cpm)

at least five days to appear and growth curves performed in parallel with induction studies indicated that cell division also reached a maximum at five days. This raised the question of whether induction by theophylline was a density-dependent phenomenon. In an initial experiment, cultures of HCT-8 Nu2 were allowed to reach confluence at which time medium either lacking or containing theophylline was placed on the cells. While counts obtained from cultures incubated for both 24 and 48 hours with the inducer were slightly higher than control cultures (Table 12), they were not 3-fold higher as had been previously observed. In a second experiment (Table 13), cells were split daily for one week to obtain duplicate cultures in various stages of confluence. On day 7, the duplicate cultures from each day were refed with either fresh medium or medium containing theophylline and tested 24 hours later. Again, the results indicated that cells contained comparable levels of cell surface CEA whether they were incubated with or without inducer. A third experiment clearly demonstrated that theophylline induction was not a density-dependent phenomenon (Table 14), as a culture incubated with theophylline for 24 hours after reaching confluence was marginally but not nearly as induced as a culture incubated with theophylline for all six days.

Table 12

Effects of Theophylline on Contact-Inhibited HCT-8 Nu2 Cells[a]

Inducer	Specific cmp[b]	
	24 hours	48 hours
None	7,033	13,527
1.5 mM theophylline	12,468	18,940

[a] Cultures were initiated on day zero with 2×10^7 cells and grown until they reached confluence at day 5. Individual cultures were refed with theophylline-containing or fresh medium and tested 24 and 48 hours later.

[b] Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

Table 13

Determination of Density-Dependence of Theophylline Induction of HCT-8 Nu2 Cells

Culture Age[a]	Inducer	Specific cpm[b]
7 days	-	65,270
	+	53,831
5 days	-	26,832
	+	39,892
4 days	-	30,121
	+	28,692
3 days	-	17,839
	+	19,827
1 day	-	12,166
	+	11,066

a Duplicate cultures were initiated daily for one week with 2×10^7 cells/culture. At the end of the week one culture from each day received fresh medium; the other received theophylline (1.5 mM) containing medium. All cultures were tested 24 hours later with the direct cell surface RiA.

b Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

Table 14

Lack of Density-Dependent of HCT-8 Nu2 Induction by Theophylline

Inducer[a]	Time with inducer	Specific cpm[b]
None	Not added	35,213
Theophylline (1.5 mM)	6 days	108,241
Theophylline (1.5 mM)	Last 24 hours	46,306

a Duplicate cultures were initiated with 2×10^7 cells. After 24 hours medium was removed and replaced with control medium or medium containing 1.5 mM theophylline. A third set of cultures received theophylline-containing medium 24 hours before the end of the experiment; at which time cells were examined for their amount of cell surface CEA by the direct RiA.

b Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

D. Effect of dbcAMP on HCT-8 Nu2 Induction

Since theophylline's major known effect is to inhibit phosphodiesterase, thereby increasing intracellular cAMP, numerous attempts were made to mimic theophylline's inducing ability with dbcAMP, a form of cAMP capable of entering cells. As can be seen in Table 15, while increasing concentrations of theophylline produced increasing amounts of measureable CEA at the cell surface on day 6, cells incubated with comparable concentrations of dbcAMP were found to possess control levels of surface CEA. Furthermore, experiments designed to observe whether a synergistic effect would occur after exposure of HCT-8 Nu2 cells to theophylline and dbcAMP indicated that the dbcAMP inhibited the degree of theophylline induction (Table 15).

A second experiment clearly demonstrated dbcAMP's inability to induce CEA synthesis in HCT-8 Nu2 cells. Results expressed in Table 16 demonstrate that while theophylline induced a 3-fold increase of CEA on day 6, various concentrations of dbcAMP were unable to increase CEA production above control levels.

Finally, the effect of dbcAMP on confluent cultures was examined, and again, various concentrations were unable to significantly increase the level of cell surface CEA (Table 17). Thus, it appears that the theophylline effect on HCT-8 Nu2 cells may occur independently from an increase in intracellular cAMP.

Table 15

Effects of Different Concentrations of Theophylline and dbcAMP on CEA Synthesis by HCT-8 Nu2[a]

Inducer	Specific cpm[b]
None	11,767
Theophylline (.25 mM)	17,185
Theophylline (.5 mM)	23,356
Theophylline (1 mM)	35,828
Theophylline (1.5 mM)	43,508
dbcAMP (.5 mM)	10,884
dbcAMP (1 mM)	8,941
Theophylline (1 mM) + dbcAMP (.5 mM)	25,183
Theophylline (1 mM) + dbcAMP (1 mM)	28,000

a Cultures of HCT-8 Nu2 were seeded with 2 x 10^7 cells and refed with medium containing indicated concentration of inducer after 24 hours. Experiment was performed 5 days later, i.e., on day 6.

b On day 6, cultures were trypsinized, counted and a direct cell surface CEA RiA was performed in duplicate. Specific CPM was calculated by subtracting the average cpm obtained with ^{125}I-normal goat IgG from average cpm obtained with ^{125}I-goat anti-CEA IgG.

Table 16

Effects of Different Concentrations of dbcAMP on CEA Synthesis by HCT-8 Nu2 Cells[a]

Inducer	Specific cpm[b]	
	Day 3	Day 6
None	8,566	10,895
Theophylline (1.5 mM)	8,078	36,346
dbcAMP (0.1 mM)	7,902	10,196
dbcAMP (1 mM)	9,753	10,918
dbcAMP (5 mM)	toxic	toxic

a Duplicate cultures of HCT-8 Nu2 cells were seeded on day zero. On day 1, medium was replaced with medium containing concentration of indicated inducer. Separate cultures were trypsinized and examined by the direct cell surface CEA RiA on day 3 and on day 6.

b Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

Table 17

Effect of dbcAMP on Confluent Cultures of HCT-8 Nu2[a]

Inducer	Specific cpm[b]	
	24 hours	48 hours
None	11,094	11,571
Theophylline (1.5 mM)	9,801	10,875
dbcAMP (0.1 mM)	11,238	12,030
dbcAMP (1 mM)	10,302	12,261
dbcAMP (2 mM)	10,938	12,930

a Cultures were initiated on day zero with 2×10^7 cells/culture. On day 5, cultures were refed with fresh medium containing inducer at indicated concentration. Cultures were examined by direct RiA 24 and 48 hours later.

b Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

E. Minimal Time Required for Incubation with Inducer

Since theophylline induction was not a density-dependent phenomenon, attempts were made to determine the minimum time the cells had to be in contact with the inducer to produce an effect. As can be seen in Table 18, results from two experiments indicated that the longer the cells were exposed to theophylline the greater the inductive effect. Hence, there does not appear to be a short time during which the cells may be triggered to produce more CEA, rather the process appears to require continued presence of the inducer.

F. Cell Selection by Theophylline

Since continual presence of inducers for six days was required to achieve maximum induction, the possibility arose that this increase might occur through active selection of high CEA producing cells by theophylline. If the concentration of theophylline used was toxic for cells which produced low amounts of CEA, cultures would result which contained only high CEA producing cells. In order to examine this possibility, cultures of HCT-8 Nu2 were induced, then split and used to seed fresh cultures which were subsequently tested after growth in normal medium. Control cultures were treated in an identical manner.

Table 18

Determination of Minimal Theophylline Induction Time

Time with inducer[a]	Specific cpm[b]	
	Experiment #1	Experiment #2
0	11,825	34,782
1 day	40,740	26,742
2	53,778	23,832
3	45,054	48,416
4	52,461	57,789
5	ND	61,807
6	73,286	63,658

[a] All cultures were initiated on day zero with 2×10^7 HCT-8 Nu2 cells. After 24 hours, all cultures were refed with control or theophylline-containing (1.5 mM) medium. Medium containing theophylline was removed at 24 hour intervals and replaced with normal medium.

[b] Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

Results from these experiments indicated that theophylline's induction of HCT-8 Nu2 cells was probably not due to cell selection and hence, was not genetically maintained, as control cultures demonstrated surface CEA levels comparable to cells which had been grown from cultures which were previously induced.

G. Determination of Protein Synthesis Requirement for Induction

Initial experiments were conducted to determine the amount of cycloheximide required to inhibit protein synthesis in HCT-8 Nu2 cells (Figure 25). In order to determine whether protein synthesis was required for theophylline inductive effect, 24 hour pulses of 25 µg/ml of cycloheximide were added to continually induced cultures during the induction period and their CEA level was compared to cultures whose protein synthesis was not inhibited. Non-induced controls were also run. As can be seen in Table 19, although all the induced cultures expressed far more CEA than non-induced controls, all cultures incubated with a 24 hour pulse of cycloheximide expressed less CEA than induced cultures whose protein synthesis was not inhibited. In a repeat of this experiment, Table 19, this effect was further shown not to be due to removal of medium, since a culture refed with fresh inducer-containing medium 24 hours before the cells were treated expressed more CEA

Figure 25

Effects of various concentrations of cylcoheximide on protein synthesis of HCT-8 Nu2 cells. Equal numbers of cells were plated in petri dishes and grown to near-confluency. Cells were exposed for 30 minutes at 37°C to medium containing various concentrations of cycloheximide. 0.1 µCi/ml of ^{3}H-amino acids (^{3}H-aa) was then added to each plate for 1 hour at 37°C. Medium was then removed, plates washed and cells were removed with 10 mM EDTA. Cells were precipitated in test tubes with TCA for 15 minutes at 4°C. Precipitate was collected on glass fiber filters which were dried and counted in a scintillation counter. Percent maximum incorporation was based on the amount of ^{3}H-aa's incorporated by cells incubated without cycloheximide.

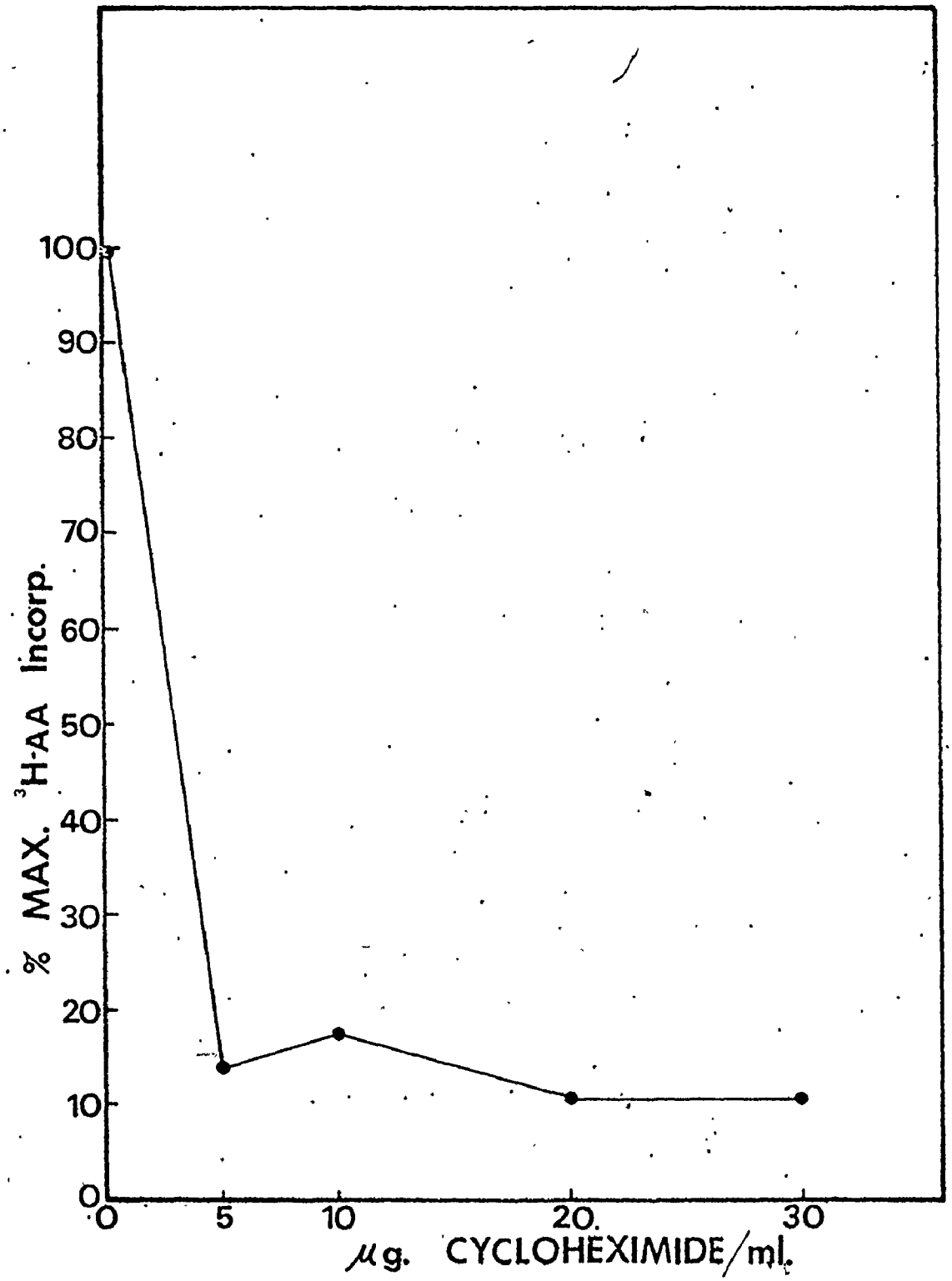

100
90
80
70
60
50
40
30
20
10
0
% MAX. ³H-AA incorp.
0
5
10
20
30
μg. CYCLOHEXIMIDE/ml.

Table 19

Inhibition of Theophylline Induction with Cycloheximide

Inducer[a]	Cycloheximide[b]	Specific cpm[c]	
		Experiment #1	Experiment #2
-	-	4,691	13,522
+	-	46,658	33,316
+	day 1	23,231	21,806
+	day 2	29,329	30,096
+	day 3	32,506	23,327
+	day 4	26,988	18,114
+	day 5	17,718	15,448
+	day 6	24,785	22,242
+[d]	-	ND	39,694

[a] 1.5 mM theophylline.

[b] 25 µg/ml of cylcoheximide in complete medium containing 1.5 mM theophylline was pulsed on the cells for a 24 hour period on the day indicated. At the end of each period cells were washed with sterile PBS and refed with medium containing inducer. All cultures initiated on day 0 and tested on day 6.

[c] Specific counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

[d] As a control, fresh inducer containing medium was placed in this culture 24 hours before testing.

than an induced culture whose medium was not changed. Hence, continual protein synthesis was required to obtain the maximum inductive effect.

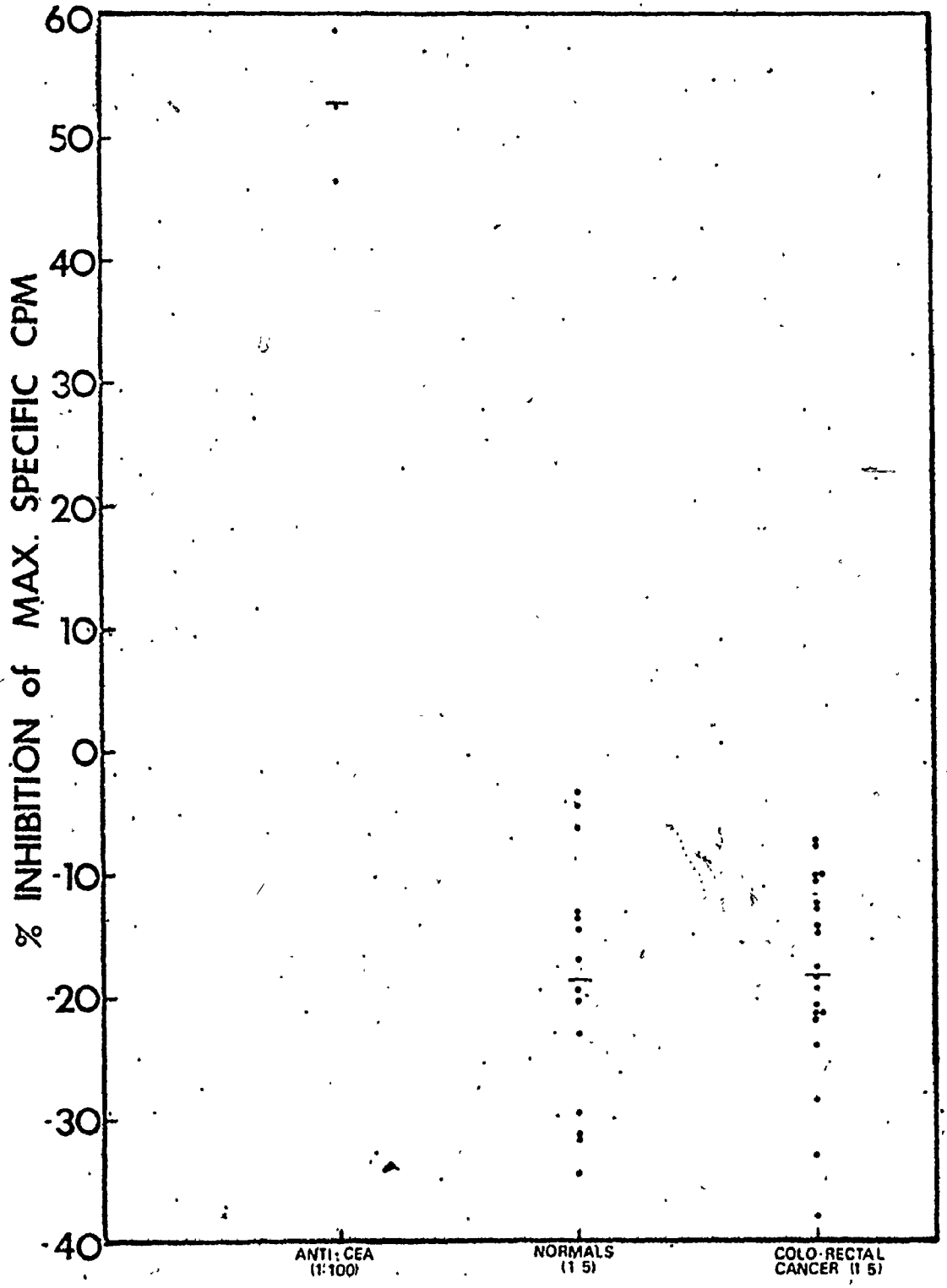
V. Clinical Significance of Carcinoembryonic Antigen

A. Test for Detection of Antibodies to Carcinoembryonic Antigen in Patient Sera

There has been disagreement concerning the question of the antigenicity of CEA in humans (Gold, 1967; Colltaz *et al.*, 1971; Gold *et al.*, 1972; MacSween, 1975). As previously indicated (Figure 15), unlabelled anti-CEA is capable of blocking the binding of ^{125}I-anti-CEA to the CEA present on the tumor cell's surface. Use was made of this reaction to determine whether antibodies to CEA exist in patient serum. In three experiments comparisons were made between serum obtained from control individuals without cancer, patients with colo-rectal cancer and a 1:100 dilution of anti-CEA. As can be seen in Figure 26, while a 1:100 dilution of anti-CEA caused a mean of 50% inhibition, absolutely no inhibition was observed with serum obtained from either control or individuals with cancer. Further, the means of negative inhibition of the two groups were identical. Thus, it appears that no antibodies to CEA can be detected in human sera.

Figure 26

Absence of detectable antibodies to CEA in human serum. 50 µl of a 1:5 dilution of serum obtained from healthy laboratory personnel or patients with colo-rectal cancer were each incubated in duplicate with 5×10^6 HCT-8R cells for 30 minutes at 24°C. Controls consisted of cells incubated with 50 µl of Tris buffer or a 1:100 dilution of goat anti-CEA. Unbound antibodies were removed by washing. 50 µl of ^{125}I-anti-CEA or ^{125}I-normal goat IgG were then added to the cells for 45 minutes at 24°C. Unbound counts were removed by washing. Specific counts per minute (cpm) were calculated by subtracting the average counts bound with ^{125}I-normal goat IgG from counts bound with ^{125}I-anti-CEA and percent inhibition was based on the maximum specific binding obtained in the absence of any serum.


% INHIBITION of MAX. SPECIFIC CPM
60
50
40
30
20
10
0
-10
-20
-30
-40
ANTI-CEA (1:100)
NORMALS (1:5)
COLO-RECTAL CANCER (1:5)

B. Determination of Dilution of Patient Serum Required to Detect Carcinoembryonic Antigen.

As previously demonstrated (Figure 5), quantities of standard CEA obtained from Hoffmann-LaRoche and an international standard of CEA dissolved in buffer were able to specifically inhibit the binding of iodine-labeled anti-CEA IgG to the antigen present on the surface of the human colon tumor cells. Furthermore, the test was capable of detecting concentrations of CEA ranging from less than 1 nanogram to greater than 16 nanograms, which is comparable to the range of detection provided by currently marketed assays.

Initial studies were performed to determine the dilution of patient serum required for detection of CEA levels. Results obtained (Table 20) indicated that undiluted serum could be used in the assay and appeared to present the most marked difference between patients with and without cancer. While an undiluted serum sample from a patient with colon cancer caused 30% inhibition of binding, a 1:4 dilution of the serum abrogated this ability. Consequently, undiluted, heat-inactivated serum was used in the assay to determine the inhibition of binding (i.e. CEA level) present in various patient sera.

Table 20

Effect of Serum Dilution on CEA Detection by Inhibition of Binding of Labeled anti-CEA to Human Colon Tumor Cell Surface CEA[a]

Serum	Dilution[b]	Specific cpm[c]	% inhibition[d]
Tris	-	11,046	0
Normal (J.G.)	undil.	12,351	0
	1:4	12,793	0
	1:8	12,926	0
Colon cancer #11	undil.	488	95.6
	1:4	5,337	51.7
	1:8	9,290	15.9
Colon cancer #14	undil.	7,741	30
	1:4	12,801	0
	1:8	11,098	0

[a] 125 µl of serum at appropriate dilution was mixed with 50 µl of ^{125}I-anti-CEA and ^{125}I-normal goat IgG for 24 hours at 37°C. Then 5 x 10^6 HCT-8R cells as 100 µl were added to each tube and allowed to react at 24°C for 45 minutes. Tubes were then washed to remove unbound counts and cells were counted in a gamma counter.

[b] Dilutions made in Tris buffer.

[c] Average counts per minute (cpm) = (Average cpm ^{125}I-anti-CEA)-(Average cpm ^{125}I-normal goat IgG).

[d] Tris tube containing no CEA is used as maximum, producing no inhibition. All percent inhibition was calculated relative to this value.

C. Comparison of our Radioimmunoassay to Hoffmann-LaRoche Assay

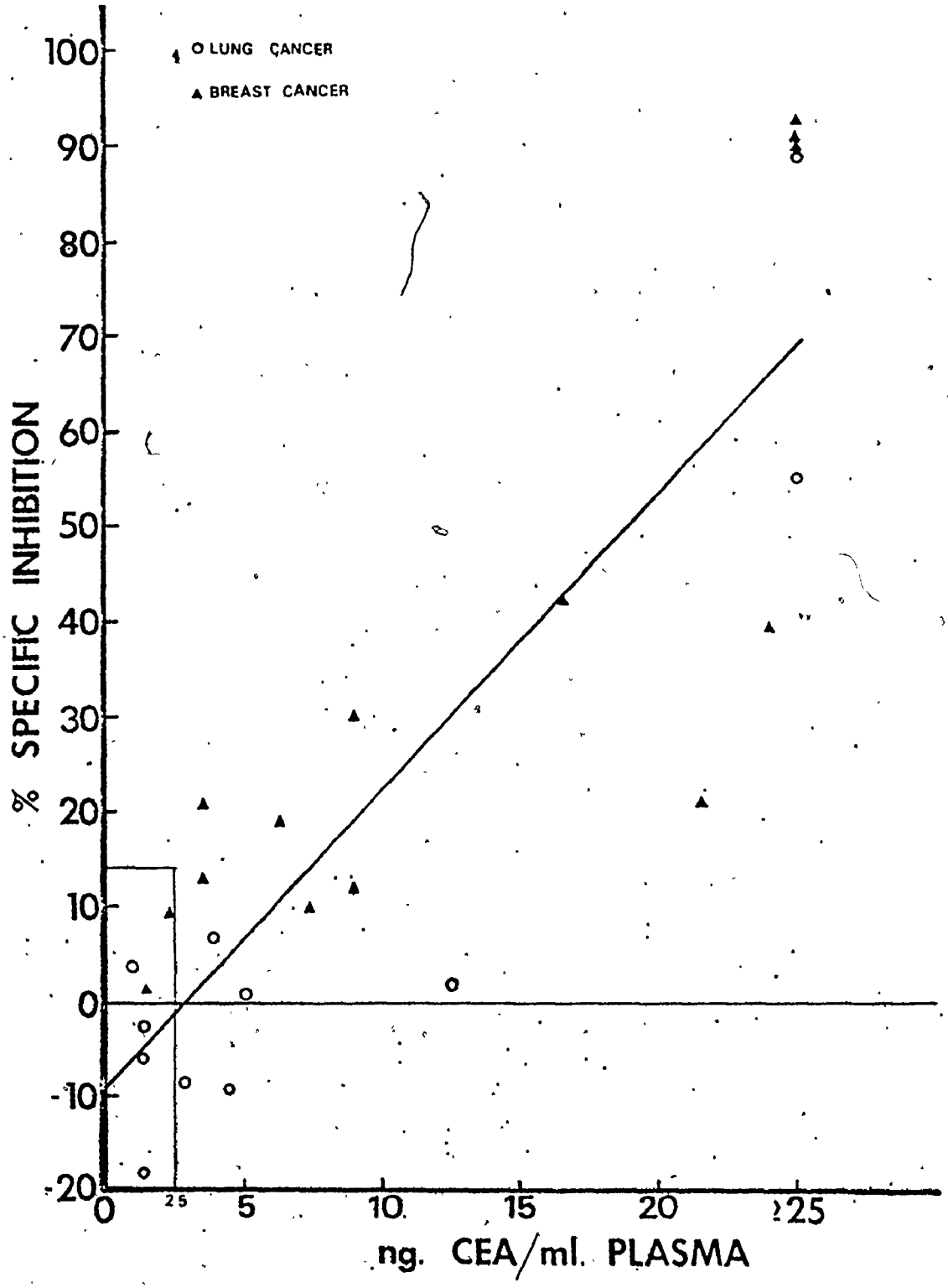
Plasma obtained from patients with breast or lung cancer were analyzed for their CEA content [in the laboratory of Dr. P. Dent by the standard Hoffmann-LaRoche assay (LoGerfo *et al.*, 1971)]. Serum obtained from the same patients were coded and examined by our RIA and the results were compared. Linear regression analysis of the data (Figure 27) indicated a correlation coefficient of 0.875 ($p < 0.001$) between the two assay procedures. Thus, the two assays appear to measure the same antigen.

D. Determination of Inter-Test Variation and at Least One Measure of "Normal"

One difficulty encountered in designing a laboratory-screening test which involves the measurement of an entity, such as CEA, lies in the problem of determining a threshold level to distinguish between "normal" and "abnormal" quantities of that entity (Sackett, 1973,1975). A number of statistical methods exist to aid in establishing this "cut-off" point. One of the most common approaches to setting the normal range is to describe it in terms of the mean value plus or minus two standard deviations. In normally distributed populations, the mean of that

Figure 27

Linear regression analysis comparing patient CEA levels detected by our assay and the Hoffmann-LaRoche assay (See text).


O LUNG CANCER
▲ BREAST CANCER
100
90
80
70
60
50
40
30
20
10
0
-10
-20
% SPECIFIC INHIBITION
0
2.5
5
10
15
20
>25
ng. CEA/ml. PLASMA

populations may be used, or one can use the mean of the control group (i.e., "normals") or the mean of the inter-test variation of one sample, since the variation of measurement contributes to the underlying biologic variation measured.

To provide a measure of inter-test variation, one individual (L.G.) without cancer (i.e., normal) was consistently run in each of the seven test serum panels examined. As can be seen in Table 21, L.G. demonstrated a mean specific percent inhibition of 30.7 ± 7.0 at one standard deviation. This indicates a coefficient of variation of 22.8% (Table 21). If all test results (see Appendix III & next section) were standardized to L.G. and L.G. was made equal to 0% or no inhibition, then greater than 14% inhibition, or two standard deviations from the mean, would represent at least one measure of "abnormal" ($p < 0.05$).

A reexamination of the data presented in Figure 27 using the calculated threshold of 14% and the established threshold of 2.5 ng CEA/ml for the Hoffmann-LaRoche assay indicate that while the two tests correlate closely there is discordance between the 2 methods with respect to the percent of sera considered positive for abnormal levels of CEA. Results obtained from our assay (Table 22) indicate a lower percentage, 32% as opposed to 76% of both breast and lung cancer patients with abnormally elevated levels of CEA.

Table 21

Calculation of Inter-Test Variation and Establishment of Normal Threshold CEA Level

Test #	% Inhibition (x)	$(\bar{x}-x)$	$(\bar{x}-x)^2$
1	34.1	3.4	11.56
2	38.7	8.0	64
3	40.3	9.6	92.16
4	23.75	7.0	49
5	28.71	2.0	4.0
6	25.3	5.4	29.16
7	24	6.7	44.89
	$\bar{x}$ = 30.69%		Σ = 294.77

Standard deviation (SD):

$$\sqrt{\frac{\Sigma(\bar{x}-x)^2}{n-1}} = \sqrt{\frac{294.77}{6}} = \sqrt{49.12} = 7.0$$

Coefficient of variation = $\frac{SD}{\bar{x}} = \frac{7.0}{30.69} = .228$ or 22.8%

Table 22

Comparison of Two CEA RIA Systems[A]

	Human colon tumor cell surface solid phase inhibition RiA		Hoffmann-LaRoche	
	no. (+)/ no. tested[b]	% (+)[c]	no. (+)/ no. tested[b]	% (+)[c]
Lung cancer	2/11	18.1	7/11	63.6
Breast cancer	6/14	42.8	12/14	85.7
Total	8/25	32.0	19/25	76.0

[A] See Figure 27.

[b] The number of patients with positive (i.e. above normal) CEA values.

[c] Percentage of patients with positive CEA values.

E. Seroepidemiologic Investigations

A series of tests were conducted using various serum panels derived from patients with and without cancer to determine the relative diagnostic applicability of the human colon tumor cell solid-phase inhibition RIA. It was anticipated that the CEA present on the surface of the colon carcinoma cells might provide a more colon cancer specific antigen to monitor.

Frequency distribution plots of the data (see Appendix III) are presented in Figure 28. As can be seen, a large percentage of colon cancer patients demonstrate high levels of CEA and, as expected, a large portion of these values fall well outside the range of CEA found in normal individuals. An attempt was made to determine whether age, sex or diagnosis of colo-rectal cancer patients correlated with markedly elevated levels of CEA. Although most patients demonstrating very high levels of inhibition had metastatic disease, there appeared to be no consistent correlation between sex, age or diagnosis and high CEA levels (Appendix IV).

The combined results of the seroepidemiologic investigation of various panels of serum obtained from individuals with and without cancer are presented in Table 23. The number and percentage of individuals possessing above "normal" levels of CEA calculated according to the

Figure 28

Frequency distribution plots demonstrating relative levels of CEA in serum obtained from patients with various conditions. See test for method and Appendix III for data calculation. The number of sera of each group studied is given in parenthesis. Distribution was calculated based on percent specific inhibition of any serum relative to L.G. Plot is condensed by a factor of 10, i.e., serum samples inhibiting 30-39.9% are plotted as 3.

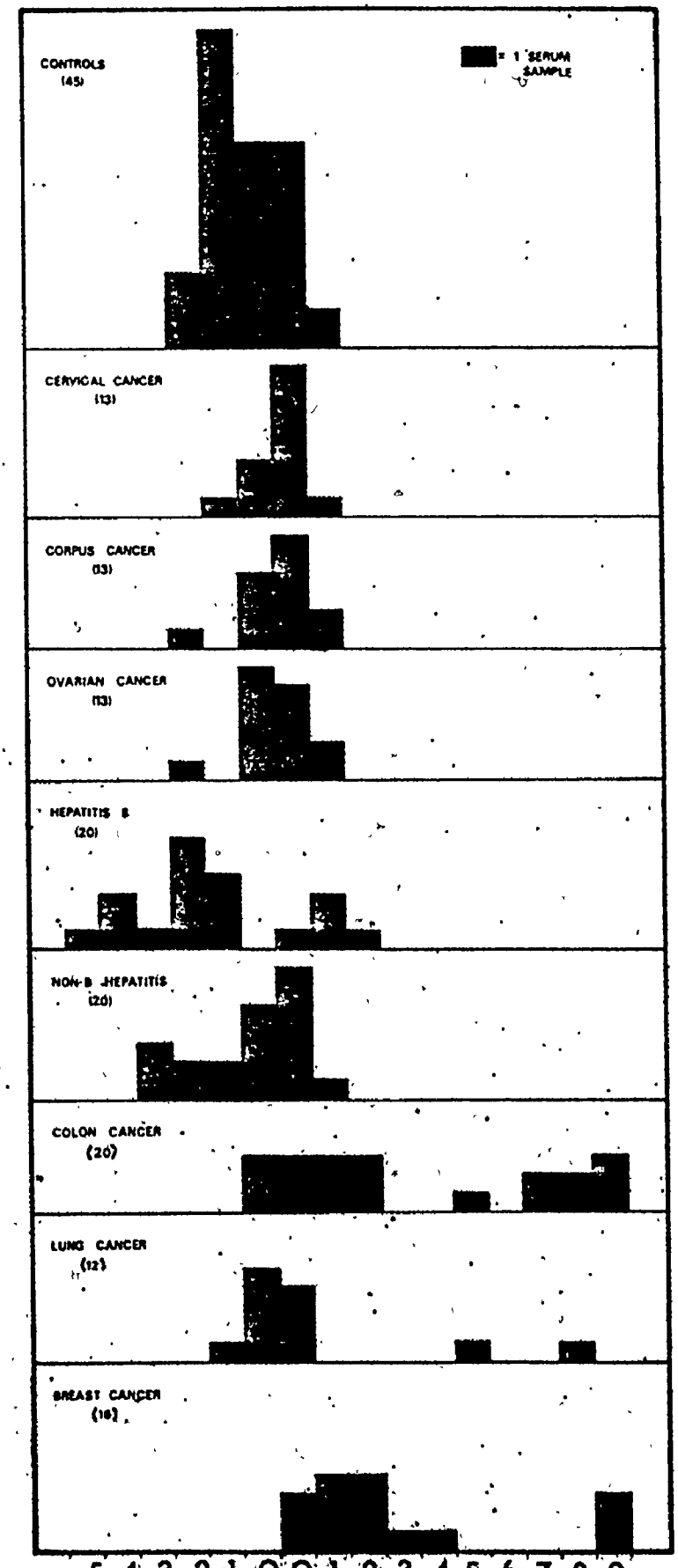

CONTROLS
(45)
= 1 SERUM SAMPLE
CERVICAL CANCER
(13)
CORPUS CANCER
(13)
OVARIAN CANCER
(13)
HEPATITIS B
(20)
NON-B HEPATITIS
(20)
COLON CANCER
(20)
LUNG CANCER
(12)
BREAST CANCER
(16)
-5 -4 -3 -2 -1 0.0 1 2 3 4 5 6 7 8 9
% SPECIFIC INHIBITION (+10)

Table 23

Summary of Seroepidemiologic Investigations[A]

	Method 1[a]		Method 2[b]		Method 3[c]	
	no. (+)/ no. tested	% (+)	no. (+)/ no. tested	% (+)	no. (+)/ no. tested	% (+)
Controls	0/45	0	0/45	0	0/45	0
Cervical cancer	0/13	0	0/13	0	0/13	0
Corpus cancer	0/13	0	0/13	0	0/13	0
Ovarian cancer	0/13	7.6	1/13	7.6	0/13	0
Colon cancer	13/20	65.0	14/20	70.0	8/20	40
Lung cancer	2/12	16.6	2/12	16.6	1/12	8.3
Breast cancer	11/16	68.8	11/16	68.8	3/16	18.8
Hepatitis B	2/20	10.0	2/20	10.0	0/20	-
Non-B-hepatitis	0/20	0	0/20	0	0/20	0

A Three methods of calculating normal range.

a) $\bar{x}$ = 0.00 ± 14 Inter-test variation at 2 standard deviations.

b) $\bar{x}$ = 7.47 ± 20.6 Mean of control group at 2 standard deviations.

c) $\bar{x}$ = 3.45 ± 53.8 Mean of total samples at 2 standard deviations.

three methods previously discussed. Method three does not hold as an efficient or proper threshold as the distribution plots demonstrate that our total population is not randomly distributed. The results indicate that this assay demonstrated a very high level of CEA positivity among colo-rectal and breast cancer patients (70%) while very few patients with other cancers were detected. No control individuals possessed high levels of CEA and only 10% of patients with hepatitis B demonstrated above "normal" levels of CEA. Thus, it appeared that our assay demonstrated a high degree of tumor specificity, detecting comparable percentages of colo-rectal cancer patients as conventional assays, yet it eliminated much of the false-positivity detected by standard marketed assays.

DISCUSSION

Fetal tissues appear to contain substances which are absent, or present, at undetectable levels in the mature organism. Some of these substances have been identified by immunological means and consequently they have been referred to as fetal antigens (Alexander, 1972). It is assumed that most fetal antigens function as "signals" or "signal receptors" required for the complex processes of differentiation and organogenesis of the developing embryo.

It is of great biological interest that malignant tumors of adult animals frequently contain high concentrations of re-expressed fetal antigens. This was clearly demonstrated by Abelev *et al.* (1963), who discovered that mouse liver tumors contained high levels of alpha feto-protein, a substance normally found only in fetal tissue. Gold & Freedman (1965a, b) similarly found that human gastrointestinal tract tumors contained substances absent from normal adult gastrointestinal tissues, but present in the developing fetal gut. Immunological tests showed that this material, which was called carcinoembryonic antigen (CEA), could be detected in the serum of patients with gastrointestinal

malignancy, but not in the serum of normal individuals or of patients with other cancers (Thompson *et al.*, 1969). The possibility that radioimmunoassays for CEA might provide a diagnostic test for gastrointestinal tract cancer provided impetus for investigations of the immunochemistry of CEA and the development and clinical use of numerous CEA assays.

The majority of studies concerning CEA have focused on the immunochemical characterization and clinical application of the molecule and it was generally agreed (Neville & Laurence, 1974) that little was known about the biology of CEA.

My investigations have used specifically labeled antibodies as probes to study the expression of CEA at the surface of human colon carcinoma cells grown *in vitro* and a radioimmunoassay for the quantitation of CEA and antibodies to CEA in the serum of cancer patients was developed.

Antiserum specific to CEA was prepared in goats and the binding of these antibodies to CEA expressed at the surface of viable colon carcinoma cells *in vitro* resulted in a rapid redistribution of the antigen to form a cap at one pole of the cell. Although, initially CEA was found to be uniformly distributed on the cell surface; if the cells were placed at 37°C the antigen-antibody complexes would migrate laterally on the cell

membrane to form a cap or protrusion at one pole. The ability of certain membrane molecules to move laterally in the plane of the membrane supports the fluid-mosaic model of membrane structure proposed by Singer & Nicolson (1972).

The polar redistribution of CEA was demonstrated by both the direct immunofluorescence test employing fluorescein-conjugated anti-CEA IgG as well as by the indirect immunofluorescence test which indicates that the primary antibody was responsible for capping. The role of anti-CEA antibodies in the polar redistribution of CEA was also demonstrated in the indirect immunofluorescence test by fixing the cells in 2.5% buffered formalin after reacting them with the primary antibodies but before the reaction with conjugated antiglobulin.

While direct and indirect immunofluorescence reactions permit visualization of cell surface reactions. They do not permit precise quantitation. For this reason, direct and indirect radiolabeled antibody assay systems were developed and optimized.

Use of iodine-125 labeled rabbit anti-goat IgG, in an indirect assay, supported fluorescent observations that the primary antibodies were responsible for capping. Further, both systems demonstrated that approximately three hours were required to completely remove CEA from the cell surface at 37°C.

As described for other antigens (Taylor *et al.*, 1971; Loor *et al.*, 1972; Joseph & Oldstone, 1974; Yahora & Edelman, 1972; Unanve & Karnovsky, 1974), CEA capping was found to be temperature-dependent and was inhibited by sodium azide and cytochalasin B. Further, protein synthesis was not required for capping, as no inhibition of capping occurred in the presence of cycloheximide.

Although the mechanism of capping has remained unclear, a number of hypotheses have been proposed to explain this phenomena. Bretscher (1976) has suggested that capping is due to a rapid, continuous, oriented flow of lipid molecules in the plasma membrane. Harris (1976) has proposed the continuous recycling of not only lipid but also integral membrane protein and carbohydrate components. In this hypothesis, capping is secondary to cell movement with directionality of membrane flow stimulated by the presence of a substratum. Other investigators have postulated an important, if not essential, role of cytoplasmic microfilaments, and/or microtubules in capping (Yahara & Edelman, 1972; Ash & Singer, 1976). These submembranous structures are believed to link cytoplasmic and membrane structures and play an active role in surface topography. Recently, Schreiner & Unanue (1977) proposed two distinct forms of capping. One form is movement-dependent and the other depends on selective interaction between membrane and cytoplasmic proteins.

Since cytochalasin B, a microfilament disrupting agent, was found to inhibit CEA capping, it would seem most likely that an interaction between the contractile apparatus of the cell and the antigen receptor on the membrane may be responsible for CEA capping.

Some authors have referred to CEA as a membrane protein while others have suggested that it is not a membrane protein but is present on the outer surface of the cell after secretion (Terry *et al.*, 1974). The fact that CEA is subject to antibody-induced redistribution suggests that the antigen should be considered a component of the plasma membrane, as are other antigens which can be capped. Furthermore these observations are compatible with CEA representing a peripheral membrane protein instead of an integral membrane protein within the fluid-mosaic model of cell membranes (Singer & Nicolson, 1972).

Studies employing indirect radiolabeled antibodies demonstrated clearly that the majority of CEA-anti-CEA complexes were endocytosed following capping. These results were supported by observations that a very small percentage of counts bound to cells reacted with iodine-labeled anti-CEA IgG were lost from these cells when the cells were incubated for prolonged periods of time under conditions favoring capping. Similar results using autoradiography (Gonatas *et al.*, 1976) and ferritin-labeled antibodies have demonstrated that internalization

follows capping of molecules at the surface of lymphocytes. Internalization of surface complexes has also been shown for neurons (Gonatas *et al.*, 1977).

Our results also indicate that CEA is continuously and rapidly regenerated at the surface of human colon carcinoma cells. Using labeled and unlabeled anti-CEA antibodies it was shown that a full complement of CEA molecules were re-expressed at the cell surface approximately three hours after the initiation of the capping reaction. The reappearance of CEA at the cell surface was shown to be temperature-dependent and required protein synthesis.

Similar studies concerning the regeneration of surface immunoglobulins on lymphocytes have demonstrated that from 2 to 4 hours were required for complete reappearance of immunoglobulin (Loor *et al.*, 1972; Wilson *et al.*, 1972). Similarly, 3 hours were required for the regeneration of surface anionic groups in mouse peritoneal macrophages (Skutelsky & Hardy, 1976).

The specificity of the various reactions described here for CEA were supported by the findings that positive reactions were not observed with cells derived from malignancies of other sites or from normal tissues. Further, cells which reacted with goat-anti-CEA did not react with normal goat serum and cells which reacted had previously been shown by radioimmunoassay to synthesize CEA (Tompkins *et al.*, 1974; Egan & Todd, 1972). Identical

reactions were also observed using our anti-CEA or a preparation of anti-CEA obtained from Dr. P. Gold. These two antisera also produced a single line of identity when reacted by Ouchterlony double diffusion with CEA. Furthermore, two standard reference sources of CEA, obtained from international and commercial sources, were both able to specifically inhibit the binding of anti-CEA antibodies to the CEA present on the surface of our colon carcinoma cells.

The specific inhibition of binding with known quantities of reference CEA also permitted an estimate of the number of CEA molecules present on an individual tumor cell. From our calculations, there was approximately 8,000 CEA molecules/cell. It was calculated that 50,000-150,000 molecules of immunoglobulin were present on an individual lymphocyte (Rabellino *et al.*, 1971).

The molecular relationship between CEA and blood group antigen A has been a subject of concern in recent years. Some authors have presented evidence that the two antigens represent different determinants on the same molecule (Gold & Gold, 1973; Holburn *et al.*, 1974) or that CEA represents modified or incomplete blood group antigens (Simmons & Perlmann, 1973). Other authors contend that CEA and blood group antigens are different molecules (Cooper *et al.*, 1974; Denk *et al.*, 1974). I found (Rosenthal *et al.*, 1975) that CEA underwent rapid

polar redistribution after binding with antibody while isoantigen A remained uniformly distributed over the cell surface after reacting with antibody. When cells which expressed both CEA and isoantigen A (HT-29) were reacted with both anti-CEA and anti-isoantigen A, the CEA capped without altering the uniform staining pattern of isoantigen A. Furthermore, incubating cells with anti-A antibodies (either IgG or IgM) before anti-CEA did not inhibit CEA redistribution while A antigen remained uniformly distributed. These findings and similar demonstrations of distinct distributions of CEA and blood group antigens observed *in vivo* (Denk *et al.*, 1974) indicate that CEA and the blood group antigens exist as separate molecules.

Although antibody-induced redistribution experiments clearly show that A antigen and CEA exist as separate molecules on the cell surface and that antibodies to CEA do not cross-react with A antigen, no conclusions can be drawn regarding cross-reaction of A antibodies with CEA. However, the failure of IgG or IgM anti-A antibodies to induce capping on HT-29 cells under any circumstances would suggest that these antibodies did not react with the CEA molecule which is readily redistributed by specific antibodies. This conclusion which is in contrast to the observation of Gold & Gold (1973) that IgM anti-A antibodies reacted with CEA is tempered by the possibility that too few anti-A antibodies are attached

to CEA to induce capping or that not all antibodies are capable of redistributing CEA.

The possibility also exists that the cross-reactions observed by others were due to new sites exposed on the soluble CEA molecules after chemical extraction. Since our work has utilized intact cell membrane-associated CEA it is possible that conformational differences exist. Another possibility is that blood group substances were contaminants of their CEA preparations. Since our source of CEA was obtained from a tumor biopsy of a blood type O patient, we did not have to concern ourselves with this problem.

A cell line (HCT-8), derived from a human adenocarcinoma of the colon, had been established in cell culture and characterized (Tompkins *et al.*, 1974). A variant was isolated from cultures of HCT-8 that differed from the parent cells in morphology, ability to grow in soft agar, marker chromosomes and CEA production (Rosenthal *et al.*, 1977). The variant cells were cloned and the strain was designated HCT-8R. This strain grew as distinct pleomorphic cells, did not form colonies in soft agar, had a 7q+ marker chromosome and produced about 2 to 3 times more CEA than the parent cells. The parent HCT-8 cells, on the other hand, grew as tightly packed colonies of epithelial cells, formed colonies in soft agar and predominantly demonstrated a 4q+ marker chromosome.

Cells from both strains were separately injected in graded doses into nude mice. Equal numbers of cells of both strains were required to form tumors in nude mice. This result suggested a lack of correlation between CEA production and oncogenicity in nude mice.

Histological examination of tumors formed by HCT-8 cells in nude mice showed areas of well-differentiated and areas of poorly differentiated adenocarcinoma. This contrasted with the uniformly poorly differentiated tumors formed by the HCT-8R strain in nude mice. These results suggested that HCT-8 cells represented a mixture of cells with different phenotypes with respect to the morphology of the cancers produced. The cloned HCT-8R cells appear to represent the more dedifferentiated phenotype.

Cell strains were established *in vitro* from tumors formed in nude mice by HCT-8 or HCT-8R, and these were designated HCT-8 Nul and HCT-8R Nul, respectively. The nude mouse passaged strains maintained the morphology and relative amount of CEA produced by their present cells. But occasional examples of the HCT-8R 7q+ marker chromosome were seen in the HCT-8 Nul strain. These results further supported the heterogeneity of the HCT-8 strain and suggested that HCT-8R-like cells composed a certain percentage of that population.

Clones were established from both HCT-8 and HCT-8R cells and examined for the quantity of cell surface CEA

they expressed. While strains derived from clones of HCT-8R demonstrated a normal distribution of high CEA production relative to HCT-8, strains derived from clones of HCT-8 demonstrated marked heterogeneity in the amount of CEA they produced. Further, it appeared that about 5% of the HCT-8 population was composed of high HCT-8R-like CEA expressor cells. These results correlate with the variability in intensity of surface staining demonstrated by immunofluorescence tests of HCT-8 cells. The recent finding of variability in the presence and quantity of Regan isoenzyme in individual cells of the HCT-8 population by Singer *et al.* (1976) supports the heterogeneity of this cell strain. Whether the apparent heterogeneity of HCT-8 was present in the original carcinoma or arose after establishing the cells in culture is not known. But the first argument is favored since the relative quantity of CEA synthesized by the various cell strains has remained stable after prolonged *in vitro* culture. Heterogeneity of an *in vivo* tumor has been hypothesized to arise through the process of tumor progression (Nowell, 1976; Prehn, 1976). This process is believed to occur through the sequential selection of mutant subpopulations which were derived from a common progenitor cell.

An apparent alteration of tumor cells was found when nude mouse passaged cell strains were reinjected

into nude mice. Both HCT-8 Nu1 and HCT-8R Nu1 cells showed a significant increase in oncogenicity with the second nude mouse transplantation. Furthermore, on the passage of HCT-8 Nu1 cells through nude mice, a line of cells (HCT-8 Nu2) was established that differed morphologically from the parent cell line. HCT-8R, on the other hand, showed neither morphological or cytogenetic changes following passage through nude mice although they became more oncogenic. The ability of cells to change their morphology and biochemical properties after passage through the nude mouse was recently demonstrated for malignant melanoma by Aubert *et al.* (1976). They noted both morphological changes and alterations in the content of 5-S-cysteinyldopa. Similar to our findings, their cells maintained the karyotype of human cells and each strain presented with the specific marker of its corresponding permanent cell line.

The association between elevated CEA production with malignant transformation makes this antigen a potential marker for evaluating differences in oncogenicity in tumor cell variants; therefore, HCT-8 and HCT-8R, as well as the nude mouse-passaged cells, were examined for membrane-associated and secreted CEA. In all cases a direct correlation was observed between the amount of cell surface CEA and CEA secreted into the culture supernatant. However, the HCT-8R variant produced

significantly more CEA than did the parent HCT-8 cell line. A single passage of either cell strain in nude mice failed to alter the level of CEA synthesis. The ability of human colon carcinoma cells to grow and maintain CEA production in nude mice has also been demonstrated by Carrel *et al.* (1976). However, a morphological variant of HCT-8 derived from the 2nd nude mouse passage showed relatively low levels of cell-associated CEA and insignificant levels in the culture supernatant. Thus, it appears possible to select for cells that produce little if any CEA. These results show no correlation between CEA production and oncogenicity for nude mice, suggesting that stimulation of CEA synthesis may not play a critical role in initiation and growth of colon carcinoma.

The effects of various inducing agents, chemicals known to alter the expression of "differentiation-associated products", on CEA production by various cell strains was examined. The HCT-8 Nu2 strain, a very low CEA producing strain, could be induced to express high levels of CEA by inclusion of theophylline in the medium. The levels of CEA produced by induced HCT-8 Nu2 cultures were comparable to levels of CEA produced by HCT-8R cells. Enhanced expression of CEA by theophylline was dose-dependent and time-dependent, requiring continual presence of the drug. The effect also required continual protein synthesis and did not cause a marked alteration of cell morphology or growth.

The major known effect of theophylline is the inhibition of phosphodiesterase activity (Robinson *et al.*, 1971). It is commonly assumed that this leads to a rise in intracellular cAMP. Cyclic AMP is known to regulate numerous cellular functions (Rev. in Rebhurn, 1977).

In these studies numerous attempts were made to mimic the theophylline effect by external addition of the dibutyrated derivative of cAMP (dbcAMP). This derivative is believed to enter cells more efficiently than cAMP (Hsie *et al.*, 1975). Under no circumstances was dbcAMP able to mimic the effect of theophylline and when the two drugs were used together the dbcAMP actually inhibited theophyllines effect. Results similar to these were recently reported by Steinberg & Whittaker (1976). They noted that theophylline would act as a potent stimulator of melanogenesis in a hamster melanoma cell line. Further, the theophylline effect on melanogenesis was diminished by dbcAMP.

Caution must be maintained in interpreting the lack of effect of dbcAMP, though, since the uptake and actual ability of this compound to increase intracellular levels of cAMP were not investigated. Further, a number of butyrated compounds are known to accumulate in cells in the presence of dbcAMP (Rebhurn, 1977) and the effects of these breakdown products are not well understood.

The enhanced expression of CEA brought about by theophylline was not density-dependent. The effect, also, could not be attributed to selection by the drug of a high CEA expressor subpopulation. Quantitation of CEA expressed by clones derived from HCT-8 Nu2 demonstrated that no high CEA expressing cells were present in this population. Further, 40% of the cells composing the HCT-8 population were HCT-8 Nu2-like with respect to the level of CEA they produced. These results support the heterogeneous nature of the HCT-8 population and also account for the weak but significant enhancement of CEA synthesis observed in the HCT-8 cells. Interestingly, the increase of CEA production in HCT-8 cells was one-third that produced in HCT-8 Nu2 cells by theophylline. Since the growth rate of cultures incubated with or without theophylline were similar, it is felt that cell selection was not the mechanism of enhancement of CEA synthesis by theophylline.

The actual mechanism of theophyllines ability to increase the expression of CEA in HCT-8 Nu2 cells is currently poorly understood. While it is generally assumed that inhibition of phosphodiesterase activity leads to a rise in intracellular cAMP, there is at least one cell line, a mouse lymphoma, in which theophylline produces no increase in cAMP content (Daniel *et al.*, 1976), and the possibility cannot be ruled out, that

theophylline has other effects on cells besides inhibiting phosphodiesterase activity. I believe that further studies on the action of theophylline as an inducer of CEA synthesis would lead to a better understanding of the control of expression of CEA.

Similar enhanced expression of CEA could be induced in HCT-8R cells with BrdU. This effect was not as dramatic as the theophylline effect on HCT-8 Nu2. The increased expression of CEA was observed transiently and did not involve a marked change in cell morphology. The effect could be produced by exposing log-phase growing cells to BrdU for 12 hours and subsequently removing it. The response to BrdU was shown to be dose-dependent. Further, this induction was not consistently reproducible and it is assumed that other factors may be involved in this phenomenon. These effects are remarkably similar to the induction of C-type particles with BrdU or other halogenated pyrimidines (Lowy *et al.*, 1971; Aaronson *et al.*, 1971).

Thus it appears that different strains of cultured human colon carcinoma cells respond to different inducing agents with increased production of CEA. This could possibly be attributed to their different degrees of differentiation. It has been postulated by Rule & Kirch (1976) that multiple genes may code for different molecules possessing CEA antigenic determinant and that these genes could be individually controlled.

Several studies have attempted to demonstrate the presence of antibodies to CEA in human sera. Sera from patients with nonmetastatic cancer of the digestive system and from pregnant women were found by Gold (1967) to agglutinate red blood cells which had been coated with a semi-purified preparation of CEA. Subsequently, Collatz *et al.* (1971) reported that using three different techniques (passive hemagglutination, immunoadsorption, and immunofluorescence) they were unable to detect antibodies to CEA. They further reported (Collatz *et al.*, 1971) that the antibodies that Gold (1967) detected were most likely directed against normal tissue components.

More recently, direct binding of immunoglobulins to radioactive-iodine labeled CEA by radioimmunoelectrophoresis was demonstrated by Gold *et al.* (1972). In some cases, this was prevented by absorbing the serum with blood group A erythrocytes, suggesting cross-reactivity by antibodies to blood group A. LoGerfo *et al.* (1972) was unable to detect any evidence of direct binding of serum globulins to ^{125}I-CEA using a 7-gel assay.

In the present study, I was unable to detect antibodies to CEA in the sera of colo-rectal cancer patients or controls. My assay involved the use of patient serum to inhibit the binding of ^{125}I-anti-CEA IgG to the CEA present on the surface of human colon carcinoma cells grown *in vitro*. This assay was shown to

be highly sensitive and specific for CEA. Further, since the cells were derived from a patient of the O blood-type (Tompkins *et al.*, 1974) and shown not to possess A blood-group antigens (Rosenthal *et al.*, 1975), the possibility of mistakenly detecting anti-blood group antibodies was eliminated.

The possibility that circulating CEA in the serum of colo-rectal cancer patients or the CEA present on their cancers would have completely removed circulating antibodies to CEA cannot be ruled out. This situation was reported, though, only to occur in patients with metastatic disease (Gold, 1967) and I have examined the sera of patients with both metastatic and nonmetastatic disease. Consequently, this possibility is made less likely. Further, since no serum from either colo-rectal cancer patients or controls were able to inhibit binding at all, it would seem that CEA is not autoantigenic.

The association of CEA with gastrointestinal tumors has been well established, and it is present at elevated concentrations in the serum of the majority of patients with adenocarcinoma of the gastrointestinal tract (Thomson *et al.*, 1969; LoGerfo *et al.*, 1971; Moore *et al.*, 1971). Although the association of CEA with digestive tract tumors has been well established, it has also been demonstrated in the serum of patients with a variety of other tumors (LoGerfo *et al.*, 1971;

Pusztaszeri & Mach, 1973; Kuo *et al.*, 1973; Laurence *et al.*, 1972). The diagnostic usefulness has been limited by the demonstration of CEA at elevated concentration in the serum of 3-18% of normal persons (Moore *et al.*, 1971) and in association with a wide variety of non-neoplastic diseases, notably liver disease, inflammatory bowel disease and chronic renal disease (Moore *et al.*, 1971a; Hansen *et al.*, 1974; Laurence *et al.*, 1972).

Contrary to the early belief that CEA represented a single homogeneous glycoprotein (Krupey *et al.*, 1968) recent studies have demonstrated biochemical heterogeneity (Gold *et al.*, 1973; Rule & Goleski-Reilly, 1974; Eveleigh, 1974; Coligan *et al.*, 1973; Banjo *et al.*, 1974a). The isoelectric profile of CEA extracted from tumors has been shown to differ from that extracted from fetal or embryonic tissues (Rule & Goleski-Reilly, 1974), and six or more molecular species of CEA-like antigens have been observed. Although the basis of this heterogenicity has not been fully established, much of it may be attributed to variations in siliac acid content (Coligan *et al.*, 1973). The observed heterogeneity introduced the possibility that CEA, as currently defined, does not represent a single molecular entity but rather a set of related isomeric glycoproteins sharing certain sets of antigenic determinants (Edgington *et al.*, 1975). Consequently, much emphasis has recently been placed on

recognizing which, if any, CEA determinants more precisely relate to the biology of tumor cells and would provide a more effective immunodiagnostic assay.

The assay system which I have designed, makes use of "natural" CEA present on the surface of human colon carcinoma cells grown *in vitro*. This CEA has not been subjected to any extraction procedures as has the soluble CEA used in most current assays. Certainly, the CEA is colon cancer specific since it is produced by colon cancer cells. Further, my assay measures the inhibition of ^{125}I-anti-CEA IgG brought about by untreated serum. Currently marketed assays often require the extraction of globulins by precipitation from patient plasma. For these reasons it was anticipated that this assay would provide a more colon cancer specific test for CEA.

In a sample of patients with carcinoma of the breast or lung, a significant correlation between my assay system and the standard Hoffmann-LaRoche assay was observed, although a markedly low incidence of elevated CEA levels was detected in these patients by my assay.

Detection of elevated serum CEA by my assay in patients with adenocarcinoma of the colon or rectum was similar to that reported using standard assays (Dhar *et al.*, 1972; Thomson *et al.*, 1969; Edgington *et al.*, 1976b). My assay also detected a fairly high percentage

of breast cancer patients with elevated CEA levels. CEA was rarely elevated though, in association with lung cancer or female urogenital neoplasms.

Although the frequency of elevation of CEA was similar in gastrointestinal cancer, as detected by my assay or standard assays, greater specificity of my assay for neoplasia was clearly evident from the study of patients without cancer. No control individuals studied were found to have elevated levels of CEA and only 10% of patients with non-B hepatitis were found with slightly elevated CEA levels. Consequently, the incidence of false-positives detected by my assay is low.

The possibility that the CEA present on the surface of the colon carcinoma cell maintained *in vitro* may represent a tumor-dominant form of CEA was supported by recent investigations of a membrane-isolated form of CEA conducted by Leung *et al.* (1977). These investigators found antigenic determinants on a membrane form of CEA which were not detectable on soluble forms of CEA. They attributed this property to a distinct conformation of membrane-associated CEA (Leung *et al.*, 1977).

The possibility that the CEA present on the tumor cells does not provide a more specific measure cannot be ruled out. For it has been noted by Zamcheck *et al.*(1975) that similar reduction of false-positive results can be

achieved in the standard assay systems available if one merely raises the amount of CEA used as the cutoff level. Certainly comparative analyses of CEA levels using my assay and standard assays must be conducted on many more patients, including more with non-neoplastic diseases and carefully staged neoplasms to establish specificity.

It is hoped that continued investigations of this type will facilitate detection and management of cancer patients.

APPENDICES

APPENDIX I

The molecular weight of CEA has been estimated to be about 180,000 (Krupey *et al.*, 1972; Coligan *et al.*, 1972). The amount of soluble CEA required to specifically inhibit binding of ^{125}I-anti-CEA by 50% is about 6 ng (Figure 5) and 2 x 6 ng = 12 ng constitutes the approximate amount required to produce 100% inhibition. This:

$$\left(\frac{12 \times 10^{-9} \text{ grams}}{18 \times 10^{4} \text{ grams/mole}}\right) \times 6.023 \times 10^{23} \text{ molecules/mole}$$

provides an estimate of the number of molecules of CEA on the cells in the reaction mixture or 4.01×10^{10}. This number divided by the total number of cells (5×10^{6}) which give the number of CEA molecules per cell: 8×10^{3} molecules of CEA/cell.

Appendix II

Calculation of Mean Ratio for Various Inducing Agents on Various Cell Lines

Number of days with inducer	Inducing agent	HEl Expt. #			HCT-8 Nu2 Expt. #				HCT-8 Expt. #				HCT-8R Expt. #			
		1	2	mean	1	2	3	mean	1	2	3	mean	1	2	3	mean
1	DEX	-.199	-.176	-.167	.595	.31	-	.452	-.188	.013	-	-.087	.072	-.083	-	-.005
	DMSO	.258	-	.258	.42	-	-	.42	-.194	-	-	-.194	-.325	-	-	-.325
	THEO	-.228	-	-.228	.128	-	.295	.211	-.142	-	.055	-.043	-.011	-	-.361	-.186
	BrdU	-.152	-.255	-.203	.272	.259	.101	.034	.042	-.13	-.011	.137	-	-.354		-.108
2	DEX	.06	-	.06	-.390	-	-	-.390	.024	-	-	.024	.086	-	-	.086
	DMSO	0.18	-	0.18	-.552	-	-	-.552	.297	-	-	.297	-.231	-	-	-.231
	THEO	.219	-	.219	-.568	-	-	-.568	.085	-	-	.085	-.03	-	-	-.03
	BrdU	-.298	-	-.298	-.584	-	-	-.584	.357	-	-	.357	.170	-	-	.17
3	DEX	-.009	0	-.004	.007	-.199	-	-.096	-.05	-.129	-	-.089	.106	-.14	-	-.017
	DMSO	-.275	0	-.137	-.275	-	-	-.275	-.228	-	-	-.228	-.475	-	-	-.475
	THEO	.011	0	.005	.041	-	.196	.096	.07	-	1.06	.566	-.239	-	-.01	-.124
	BrdU	.098	0	.049	.169	.512	.151	.152	-.034	.219	.273	.152	.65	-	1.18	.913
4	DEX	-.182	-.154	-.168	.138	.172	-	.155	-.455	-.078	-	-.266	-.063	-.025	-	-.044
	DMSO	-	-	-	-.341	-	-	-.341	-.712	-	-	-.712	-.089	-	-	-.089
	THEO	-.443	-	.443	2.060	-	-	2.060	.324	-	-	.324	-.115	-	-	-.115
	BrdU	-.254	-.148	-.201	.003	.256	-	.129	-.065	.055	-	-.005	.088	-	-	.088
5	DEX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	THEO	-	-	-	-	-	1.734	1.734	-	-	.835	.875	-	-	-.023	-.023
	BrdU	-	-	-	-	-	-.062	-.062	-	-	-.121	-.121	-	-	.653	.653
6	DEX	.643	0.317	.163	.559	.121	-	.34	-.02	.136	-	.058	.172	-.063	-	.054
	DMSO	-.02	-	-.02	-.335	-	-	-.335	-.51	-	-	-.51	0.48	-	-	-.48
	THEO	-	-	-	2.364	-	2.342	.844	-	1.176	1.01	-.038	-	.332		.347
	BrdU	-.125	.551	.231	.150	1.169	-0.43	.425	-.106	.310	.255	.153	.063	-	.614	.637

A See text for method

APPENDIX III

Calculations from Seroepidemiologic Investigations[a]

	Experiment 1			Experiment 2		
	Specific cpm	% maximum	% L.G.	Specific cpm	% maximum	% L.G.
Tris	23,548	0	-51.7	31,134	0	-63.0
L.G.	15,521	34.1	0	19,089	38.7	0
NUNS	18,337	22.2	-18.1	22,054	29.2	-15.5
2	17,968	23.7	-15.7	21,780	30.1	-14.0
3	17,903	24.0	-15.3	21,885	29.8	-14.6
4	19,449	17.5	-25.3	19,725	36.7	- 3.3
5	18,917	19.7	-21.8	21,878	29.8	-14.6
6	19,166	18.7	-23.4	23,356	25.0	-22.3
7	18,097	23.2	-16.5	22,736	27.0	-19.1
8	17,168	27.1	-10.6	22,764	27.0	-19.2
9	16,098	31.7	- 3.7	21,364	31.4	-11.9
10	18,232	22.6	-17.4	22,227	28.7	-16.4
LAB	14,684	37.7	5.4	20,183	35.2	- 5.7
2	15,683	33.4	- 1.0	19,008	39.0	0.5
3	14,499	38.5	6.6	20,256	35.0	- 6.1
4	15,905	32.5	- 2.4	20,914	32.9	- 9.5
5	15,282	35.2	1.6	18,698	40.0	2.1
6	14,639	37.9	5.7	21,300	31.6	-11.5
Colon Ca	4,497	81.0	71.1	14,763	52.2	22.7
2	3,465	85.3	77.7	13,669	56.1	28.4
3	13,192	44.0	15.1	2,873	90.8	85.0
4	746	96.9	95.2	18,345	41.1	3.9
5	13,448	42.9	13.4	17,976	42.3	5.9
6	1,122	95.3	92.8	19,723	36.7	- 3.3
7	16,566	29.7	- 6.7	20,831	33.1	- 9.1
8	14,269	39.5	8.1	1,692	94.6	91.2
9	6,343	73.1	59.2	2,018	93.6	89.6
10	11,110	52.9	28.5	16,320	47.6	14.6

[a] See text for method.

APPENDIX III (cont'd)

	Experiment 3				Experiment 4		
	Specific cpm	% maximum	% L.G.		Specific cpm	% maximum	% L.G.
Tris	36,611	0	-67.3	Tris	35,406	0	-31.1
L.G.	21,874	40.3	0	L.G.	27,000	23.8	0
Controls	22,950	37.4	- 4.9	Corpus Ca.	28,198	20.4	- 4.4
2	23,746	35.2	- 8.5	2	27,088	23.5	- 0.3
3	22,088	39.7	- 0.9	3	26,113	26.3	3.3
4	19,747	46.1	9.8	4	26,771	24.4	0.9
5	19,360	47.2	11.5	5	26,590	25.0	1.6
6	21,168	42.2	3.3	6	23,783	32.9	12.0
7	26,233	28.4	-19.9	7	26,278	25.8	2.7
8	19,149	47.7	12.5	8	27,191	23.3	- 0.7
9	22,311	39.1	- 1.9	9	24,255	31.5	10.2
10	21,578	41.1	1.4	10	25,919	26.8	4.1
11	21,157	42.3	3.3	11	25,222	28.8	6.6
12	24,826	32.2	-13.4	12	38,843	1.6	-29.0
13	20,974	42.8	4.2	13	29,407	17.0	- 8.9
Cervical Ca.	22,280	39.2	- 1.8	Ovarian Ca.	28,123	20.6	- 4.1
2	24,873	32.1	-13.7	2	24,181	31.8	10.5
3	21,291	41.9	2.7	3	29,495	16.7	- 9.2
4	22,063	39.8	- 0.8	4	27,162	23.3	- 0.6
5	19,088	47.9	12.8	5	26,479	25.3	2.0
6	20,0331	44.5	7.1	6	22,736	35.8	15.8
7	19,843	45.8	9.3	7	28,324	20.1	- 4.9
8	20,646	43.7	5.7	8	26,880	24.1	0.5
9	19,744	46.1	9.8	9	26,270	25.9	2.8
10	21,844	40.4	0.2	10	28,121	20.6	- 4.1
11	22,009	39.9	- 0.6	11	25,705	27.4	4.8
12	20,990	42.7	4.1	12	27,732	21.7	- 2.7
13	21,723	40.7	0.7	13	34,283	3.2	-26.9

APPENDIX III (cont'd)

	Experiment 5		
	Specific cpm	% maximum	% L.G.
Tris	26,114	0	-40.2
L.G.	18,619	28.8	0
Lung Ca.	19,615	24.9	- 5.3
2	2,033	92.3	89.1
3	19,716	24.6	- 5.8
4	22,023	15.7	-18.2
5	20,433	21.8	- 9.7
6	18,333	29.8	1.6
7	20,222	22.6	- 8.6
8	8,367	68.0	55.1
9	19,150	26.7	- 2.8
10	17,422	33.3	6.5
11	18,508	29.2	0.6
12	17,981	31.2	3.5
Breast Ca.	14,756	43.5	20.8
2	10,786	58.7	42.1
3	15,175	37.8	18.5
4	16,244	41.9	12.8
5	13,104	49.9	29.7
6	16,823	35.6	9.7
7	16,003	38.8	14.1
8	13,190	49.5	29.2
9	11,305	56.8	39.3
10	1,350	94.9	92.8
11	1,814	93.1	90.3
12	16,947	35.2	9.0
13	18,434	29.5	1.0
14	1,867	92.9	90.0
15	16,471	37.0	11.6
16	14,818	43.3	20.5

APPENDIX III (cont'd)

	Experiment 6				Experiment 7		
	Specific cpm	% maximum	% L.G.		Specific cpm	% maximum	% L.G.
Tris	27,801	0	-33.8	Tris	49,618	0	-31.5
L.G.	20,769	25.3	0	L.G.	37,711	24.0	0
LAB (-)	22,964	17.5	-10.5	LAB	41,948	15.5	-11.2
Colon Ca.(+)	2,414	91.4	88.4	Colon Ca.(+)	2,157	95.7	94.3
2	2,671	90.4	87.2	2	5,080	89.8	86.6
HEP.B	26,178	5.9	-26.0	HEP.B	42,293	14.8	-12.1
2	18,537	33.4	10.8	2	43,614	12.2	-15.6
3	25,891	6.9	-24.6	3	42,820	13.8	-13.5
4	29,468	- 5.9	-41.8	4	31,492	36.6	16.5
5	31,642	-13.8	-52.3	NON.B	45,015	9.3	-19.3
6	30,767	-10.6	-48.1	2	38,040	23.4	- 0.8
7	30,118	- 8.3	-45.0	3	39,499	20.4	- 4.7
8	25,176	9.5	-21.2	4	42,018	15.4	-11.4
9	27,587	0.8	-32.8	5	34,541	30.4	8.5
10	26,423	5.0	-27.2	6	45,915	7.5	-21.7
11	20,096	27.8	3.3	7	34,372	30.8	8.9
12	15,502	44.3	25.4	8	38,624	22.2	- 2.4
13	25,070	9.9	-20.7	9	39,638	20.2	- 5.1
14	18,571	33.2	10.6	10	37,558	24.4	0.5
15	23,724	14.7	-14.2	11	37,011	25.5	1.9
16	25,912	6.8	-24.7	12	35,629	28.2	5.6
NON.B	20,205	27.4	2.8	13	33,708	32.1	10.7
2	21,170	23.9	- 1.9				
3	20,478	26.4	1.5				
4	27,694	0.4	-33.3				
5	26,733	3.9	-28.7				
6	27,860	- 0.2	-34.1				
7	27,853	- 0.1	-34.1				

APPENDIX IV

Correlation Between Sex, Age or Diagnosis and Serum Carcinoembryonic Antigen Level

Patient #	Sex	Age	Metastasis or recurrence	Diagnosis	Specific inhibition
1	F	48	M	Adenocarcinoma of the signoid colon	30
2	F	55	M	Adenocarcinoma of the uterus	34
3	F	60	R	Adenocarcinoma of the rectum (poor differentiation)	37
4	M	61	R	Adenocarcinoma of the rectum	40
5	F	59	M	Adenocarcinoma of the colon	42
6	F	48	M	Carcinoma of the colon (well differentiated)	43
7	F	64	M	Adenocarcinoma of the rectum	43
8	F	84	-	Carcinoma of the rectum	44
9	F	73	M	Adenocarcinoma of the colon	48
10	M	66	M	Carcinoma of the rectum (well differentiated)	53
11	F	85	M	Adenocarcinoma of the rectum	57
12	F	71	M	Adenocarcinoma of the colon (well differentiated)	74
13	M	28	-	Adenocarcinoma of the rectum	81
14	F	58	-	Adenocarcinoma of the signoid colon (Stage II)	86
15	M	62	M	Adenocarcinoma of the rectum	91
16	F	62	M	Adenocarcinoma of the colon	94
17	F	79	M	Adenocarcinoma of the colon	95
18	M	59	M	Carcinoma of the rectum (well differentiated)	96
19	M	56	M	Carcinoma of the colon (well differentiated)	97

REFERENCES

Aaronson, J.A., Todaro, G.J. & Scolnick, E.M. (1971).
Induction of murine C-type viruses from clonal lines of virus free BALB/3T3 cells.
Science 174, 157-159.

Abelev, G.I., Detrova, S.D. & Khramkova, N.I. (1963).
Production of embryonal α-globulin by transplantable mouse hepatoma.
Transplantation 1, 174-198.

Abelev, G.I. (1971).
Alpha-fetoprotein in oncogenesis and its association with malignant tumors.
Adv. Cancer Res. 14, 295-358.

Abelev, G.I. (1974).
Alpha-fetoprotein as a marker of embryo-specific differentiation in normal and tumor tissue.
Transpl. Rev. 20, 3-37.

Alexander, P. (1972).
Fetal antigens in cancer.
Nature 235, 137-181.

Anderson, N.G. & Coggin, J.H. (1974).
The interrelations between development, retrogenesis, viral transformation and human cancer.
IN: *Cell Surface in Development* (A.A. Moscona, ed.) Wiley Publishers, pp. 297-314.

Ash, J.F. & Singer S.J. (1976).
Concanavalin A-induced transmembrane linkage of Concanavalin A surface receptors to intracellular myosin-containing filaments.
Proc. Nat. Acad. Sci. USA 73, 4575-4579.

Aubert, C., Chirieceanu, E.; Foa, C., Rorsman, H., Rosengren, E. & Rouge F. (1976).
Ultrastructual and biochemical changes in cultured human malignant melanoma cells after heterotransplantation into nude mice.
Cancer Res. 36, 3106-3112.

Bagshawe, K.D., Rogers, G.T., Searle, F. & Wilson, H. (1973).
Blood carcinoembryonic antigen, Regan isoenzyme and human chorionic gonadotrophin in primary mediastinal carcinoma.
Lancet 1, 210-211.

Baldwin, R.W., Embleton, M.J., Price, M.R. & Vose, B.M. (1974).
Embryonic antigen expression on experimental rat tumors.
Transpl. Rev. 20, 77-99.

Banjo, C., Gold, P., Gehike, C.W., Freedman, S.O. & Krupey, J. (1974).
Preparation and isolation of immunologically active glycopeptides from carcinoembryonic antigen.
Int. J. Cancer 13, 151-163.

Banjo, C., Shuster, J. & Gold, P. (1974a).
Intermolecular heterogeneity of the carcinoembryonic antigen.
Cancer Res. 34, 2114-2121.

Bern, H.A. & Nandi, S. (1961).
Recent studies of the hormonal influence in mouse mammary tumorigenesis.
Progr. Exptl. Tumor Res. 2, 90-144.

Bienenstock, J. & Strauss, H.J. (1970).
Evidence for synthesis of human colostral IgA as 11 S dimer.
J. Immunol. 165, 274-277.

Braun, A.C. (1975).
Differentiation and dedifferentiation.
IN: *Cancer* Vol. 3 (F.F. Becker, ed.) Plenum Press, N.Y. pp. 3-20.

Bretscher, M.S. (1976).
Directed lipid flow in cell membranes.
Nature 260, 21-23.

Burtin, P., Sabine, M.C. & Chavanel, G. (1972).
Presence of carcinoembryonic antigen in childrens colonic mucosa.
Int. J. Cancer 10, 72-76.

Burtin, P., Chavanel, G. & Hirsch-Marie, H. (1973).
Characterization of a second normal antigen that cross-reacts with CEA.
J. Immunol. 111, 1926-1928.

Carrel, S., Sordat, B. & Merenda, C. (1976).
Establishment of a cell line (Co-115) from a human colon carcinoma transplanted into nude mice.
Cancer Res. 36, 3978-3984.

Cherry, W.B. (1974).
Immunofluorescence techniques.
IN: *Manual of Clinical Microbiology* (E.H. Lennette, E.H. Spaulding and J.P. Truant, eds.) 2nd ed. American Society for Microbiology, Washington, D.C. pp. 29-44.

Coggin, J.H., Ambrose, K.R., Bellomy, B.B. & Anderson, N.G. (1971).
Tumor immunity in hamsters immunized with fetal tissues.
J. Immunol. 107, 526-533.

Coggin, J.H. & Anderson, N.G. (1974).
Cancer, differentiation and embryonic antigens: Some central problems.
Adv. Cancer Res. 19, 105-166.

Coligan, J.E., Lavtenschleger, J.T., Egan, M.L. & Todd, C.W. (1972).
Isolation and characterization of carcinoembryonic antigen.
Immunochem. 9, 377-386.

Coligan, J.E., Henkart, P.A., Todd, C.W. & Terry, W.P. (1973).
Heterogeneity of the carcinoembryonic antigen.
Immunochem. 10, 591-599.

Coligan, J.E. & Todd, C.W. (1975).
Structural studies on carcinoembryonic antigen periodate oxidation.
Biochem. 14, 805-810.

Coligan, J.E., Pritchard, D.G., Schnute, W.C. & Todd, C.W. (1976).
Methylation analysis of the carbohydrate portion of carcinoembryonic antigen.
Cancer Res. 36, 1915-1917.

Collatz, E., von Kleist, S. & Burtin, P. (1971).
Further investigations of circulating antibodies in colon cancer patients: On the autoantigenicity of the carcinoembryonic antigen.
Int. J. Cancer 8, 298-303.

Comoglio, P.M. & Guglielmone, R. (1972).
Two dimensional distribution of concanavalin A receptor molecules on fibroblast and lymphocyte plasma membranes.
FEBS Letters 27, 256-258.

Cooper, A.G., Grown, M.C., Kirch, M.E. & Rule, A.H. (1974).
Relationship of CEA to blood group substances A and i: Evidence that the antigenic sites are on different molecules.
J. Immunol. 113, 1246-1251.

Costanza, M.E., Schwartz, R.S., Nathanson, L. & Patterson J.F. (1973).
Carcinoembryonic antigen: A preliminary report of a long term prospective study.
Cancer Chemother. Rep. 57, 105.

Costanza, M.E. & Nathanson, L. (1974).
Carcinofetal antigens.
Progr. Clin. Immunol. 2, 191-224.

Daniel, V.H., Bourne, R. & Tompkins, G.M. (1973).
Altered metabolism and endogenous cyclic AMP in cultured cells deficient in cyclic AMP binding protein.
Nature New Biol. 244, 167-169.

Darcy, D.A., Tuberville, C. & James, R. (1973).
Immunological study of carcinoembryonic antigen and a related glycoprotein.
Br. J. Cancer 28, 147-160.

Davidson, E.H. & Britten, R.J. (1974).
Molecular aspects of gene regulation in animal cells.
Cancer Res. 34, 2034-2043.

Denk, H., Tappeiner, G., Davidovits, A., Eckerstorfer, R. & Holzner, J.H. (1974).
CEA and blood group substances in carcinomas of the stomach and colon.
J. Nat. Cancer Inst. 53, 933-938.

Dhar, P., Moore, T.L., Zamcheck, N. & Kupchik, H.Z. (1972).
Carcinoembryonic antigen in colonic cancer: Use in pre and postoperative diagnosis and prognosis.
J. Amer. Med. Assoc. 221, 31-35.

Dykes, P.W., Booth, S.N. & King J. (1974).
CEA is a prognostic index in colorectal cancer.
Abstracts of the Fifth World Congress of Gastroenterology, Mexico City, p. 320.

Edgington, T.S., Astarita, R.W. & Plow, E.F. (1975).
Association of an isomeric species of carcinoembryonic antigen with neoplasia of the gastrointestinal tract.
New Eng. J. Med. 293, 103-107.

Edgington, T.S., Plow, E.F., Go, W., Herberman, R., Burtin, P., Jordan, I., Chavkin, C., DeHeer, D.H. & Nakamura, R.M. (1976a).
A comparison of CEA-5 and CEA concentrations in sera and the independence of CEA-5, NCA and blood group antigens.
Bull. du Cancer 63, 613-626.

Edgington, T.S., Plow, E.F., Chavkin, C.I., DeHeer, D.T. & Nakamura, R.M. (1976b).
The influence of CEA-5 from different tumors and of CAS as ^{125}I ligands on the specificity of the CEA-5 radioimmunoassay.
Bull. du Cancer 63, 673-688.

Egan, M.L. & Todd, C.W. (1972).
Carcinoembryonic antigen: Synthesis by a continuous line of adenocarcinoma cells.
J. Nat. Cancer Inst. 49, 887-889.

Egan, M.L., Lautenschleger, J.T., Coligan, J.E. & Todd, C.W. (1972).
Radioimmunne assay of carcinoembryonic antigen.
Immunochem. 9, 289-299.

Egan, M.L., Coligan, J.E. & Todd, C.W. (1974).
Radioimmune assay - Principles and practical applications
(E. Haber, ed.) Little, Brown & Co., Boston.

Egan, M.L., Coligan, J.E., Pritchard, D.G., Schnute, W.C. & Todd, C.W. (1976).
Physical characterization and structural studies of the carcinoembryonic antigen.
Cancer Res. 36, 3482-3485.

Elias, E.G., Holyoke, E.D. & Chu, T.M. (1974).
Carcinoembryonic antigen in feces and plasma of normal subjects and patients with colorectal carcinoma.
Dis. Colon Rectum 17, 38-41.

Eveleigh, J.W. (1974).
Heterogeneity of the carcinoembryonic antigen.
Cancer Res. 34, 2122-2124.

Friend, C., Scher, W., Holland, J.G. & Sato, T. (1971)
Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*.
Proc. Nat. Acad. Sci. USA 68, 378-382.

Frye, L.P. & Edidin, M.J. (1970).
The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons.
J. Cell Sci. 7, 319-354.

Fuks, A., Banjo, C., Shuster, J., Freedman, S.O. & Gold, P. (1975).
Carcinoembryonic antigen: Molecular biology and clinical significance.
Biochim. Biophys. Acta 417, 123-152.

Gold, J.M., Freedman, S.O. & Gold, P. (1972).
Human anti-CEA antibodies detected by radioimmuno-electrophoresis.
Nature New Biol. 239, 60-62.

Gold, J.M., & Gold P. (1973).
The blood group A-like site on the carcinoembryonic antigen.
Cancer Res. 33, 2821-2824.

Gold, J.M., Banjo, C., Freedman, S.O. & Gold, P.(1973).
Immunochemical studies of the intramolecular heterogeneity of the carcinoembryonic antigen of the human digestive system.
J. Immunol. 111, 1872-1879.

Gold, P. & Freedman, S.O. (1965a).
Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques.
J. Exp. Med. 121: 439-462.

Gold, P. & Freedman, S.O. (1965b).
Specific carcinoembryonic antigens of the human digestive system.
J. Exp. Med. 122, 467-481.

Gold, P. (1967).
Circulating antibodies against carcinoembryonic antigens of the human digestive system.
Cancer 20, 1663-1667.

Gold, P., Gold, M. & Freedman, S.O. (1968).
Cellular location of carcinoembryonic antigen of the human digestive system.
Cancer Res. 28, 1331-1334.

Gold, P., Krupey, J. & Ansari, H. (1970).
Position of the carcinoembryonic antigen of the human digestive system in ultrastructure of tumor cell surface.
J. Nat. Cancer Inst. 45, 219-225.

Gonatas, N.K., Gonatas, J.O., Stieber, A., Antoine, J.C. & Aurameas, S. (1976).
Quantitative ultrastructural autoradiographic studies of iodinated plasma membranes of lymphocytes during segregation and internalization of surface immunoglobulins.
J. Cell Biol. 70, 477-493.

Gonatas, N.K., Kim, S.U., Stieber, A. & Aurameas, S. (1977).
Internalization of lectins in neuronal gel.
J. Cell Biol. 73, 1-13.

Gusella, J., Geller, R., Clarke, B., Weeks, V. & Housman, D. (1976).
Commitment to erythroid differentiation by Friend erythroleukemic cells: A stochastic analysis.
Cell 9, 221-229.

Häkkinen, I. (1972).
Immunological relationship of the carcinoembryonic antigen and the fetal sulfoglycoprotein antigen.
Immunochem. 9, 1115-1119.

Hammerström, S., Enguall, E., Johansson, B.G., Svenson, S., Sundblad, G. & Goldstein, I.J. (1975).
Nature of the tumor-associated determinants of carcinoembryonic antigen.
Proc. Nat. Acad. Sci. USA 72, 1528-1532.

Hansen, H.J., Lance, K.P. & Krupey, J. (1971).
Demonstration of an ion sensitive antigenic site on carcinoembryonic antigen using zirconyl phosphate.
Clin. Res. 19, 143.

Hansen, H.J., Snyder, J.J., Miller, E., van der Voord, J.P., Miller, O.N., Hires, L.R. & Burns, J.J. (1974).
Carcinoembryonic antigen assay: A laboratory adjunct in the diagnosis and management of cancer.
Hum. Pathol. 5, 139-147.

Harris, A.K. (1976).
Recycling of dissolved plasma membrane components as an explanation of the capping phenomenon.
Nature 263, 781-783.

Harvey, S.R. & Chu, T.M. (1975).
Demonstration of two molecular variants of carcino-embryonic antigen by concanavalin A sepharose affinity chromatography.
Cancer Res. 35, 3001-3008.

Hayflick, L. (1965).
Tissue cultures and mycoplasmas.
Texas Rept. Biol. Med., 23 (Suppl. 1), 285-303.

Hellstrom, I. & Hellstrom, K.E. (1975).
Cytotoxic effect on lymphocytes from pregnant mice on cultivated tumor cells.
Int. J. Cancer 15, 1-6.

Helson, L., Lai, K. & Yound, C.W. (1974).
Papaverine-induced changes in cultured human melanoma cells.
Biochem. Pharmacol. 23, 2917-2920.

Herberman, R.B., Aoki, T., Cannon, G., Liu, M. & Sturm, M. (1975).
Location by immunoelectron microscopy of carcino-embryonic antigen on cultured adenocarcinoma cells.
J. Nat. Cancer Inst. 55, 797-798.

Hirszfeld, L., Halber, W. & Laskowski, J. (1929).
Untersuchungen uber serologische eigenschaften der Gewebe.
Z. Immunitaetsforsch. 64, 81-113.

Holburn, A.M., Mach, J.P. & McDonald, D. (1974).
Studies of the association of the A,B and Lewis blood group antigens with carcinoembryonic antigen.
Immunol. 26, 831-843.

Hollinshead, A., Glew, P., Bunnacy, B., Gold, P. & Herberman, R. (1970).
Skin-reactive soluble antigen from intestinal cancer-cell-membranes and relationship to carcino-embryonic antigens.
Lancet 1, 1191-1195.

Holyoke, D., Reynoso, G. & Chu, T.M. (1972).
Carcinoembryonic antigen in patients with carcinoma of the digestive tract.
Ann. Surg. 176, 559-564.

Holyoke, E.D. (1974).
Carcinoembryonic antigen in evaluation of diagnosis and treatment of surgical gastrointestinal disease.
Abstract, Fifth World Congress of Gastroenterology, Mexico City, p. 18.

Holyoke, E.D. (1975).
Present and probable uses of CEA.
Ca.-A Cancer J. for Clinicians 25, 22-26.

Holyoke, E.D., Chu, T.M. & Murphy, G.P. (1975).
Tumor-associated antigens.
Transpl. Proc. 7, 291-295.

Hsie, A.W., Kawashima, K., O'Neill, J.P. & Schroder, C.H. (1975).
Possible role of adenosine cyclic 3':5'-monophosphate phosphodiesterase in the morphological transformation of Chinese hamster ovary cells mediated by N^6,O^2-dibutyryl adenosine cyclic 3':5' monophosphate.
J. Biol. Chem. 250, 984-995.

Jacob, F. & Monod, J. (1961).
Genetic regulatory mechanisms in the synthesis of proteins.
J. Mol. Biol. 3, 318-328.

Johnson, G.S. & Pastar, I. (1972).
Dibutyrl adenosine monophosphate induces pigment production in melanoma cells.
Nature New Biol. 237, 267-268.

Joseph, B.S. & Oldstone, M.B.A. (1974).
Antibody-induced redistribution of measles virus antigens on the cell surface.
J. Immunol. 113, 1205-1209.

Kourilsky, F.M., Silvestre, D., Neuport-Sautes, C., Goosfelt, Y. & Daussert, J. (1972).
Antibody-induced redistribution of HL-A antigens at the cell surface.
Eur. J. Immunol. 2, 249-257.

Krupey, J., Gold, P. & Freedman, J.O. (1968).
Physiochemical studies of the carcinoembryonic antigens of the human digestive system.
J. Exp. Med. 128, 387-398.

Krupey, J., Wilson, T., Freedman, S.O. & Gold, P. (1972).
The preparation of purified carcinoembryonic antigen of the human digestive system from large quantities of tumor tissue.
Immunochem. 9, 617-622.

Kuo, T.T., Rosai, J. & Tillack, T.W. (1973).
Immunological studies of membrane glycoproteins from human breast carcinoma.
Int. J. Cancer 12, 532-542.

Laurence, D.J.R., Stevens, H., Bettelheim, R., Darcy, D., Leese, C., Tuberville, C., Alexander, P., Johns, E.W. & Neville, A.M. (1972).
Evaluation of the role of plasma carcinoembryonic antigen in the diagnosis of gastro-intestinal, mammary and bronchial carcinoma.
Brit. Med. J. 3, 605-611.

Laurence, D.J.R., Tuberville, C., Anderson, S.G. & Neville, A.M. (1975).
First British standard for carcinoembryonic antigen.
Brit. J. Cancer 32, 295-299.

Lausch, R.N. & Rapp, F. (1974).
Tumor-specific antigens and reexpression of fetal antigens in mammalian cells.
Progr. exp. Tumor Res. 19, 45-58.

Leung, J.P., Plow, E.F., Eshdat, Y., Marchesi, V.T. & Edgington, T.S. (1977).
Delineation of three classes of CEA antigenic determinants: Identification of membrane-associated CEA as an independent species of CEA.
J. Immunol. 119, 271-276.

Lin, C.C., Uchida, I.A. & Byrens, E.A. (1971).
Suggestion for the nomenclature of the fluorescent banding patterns in human metaphase chromosomes.
Can. J. Genet. Cytol. 13, 361-363.

LoGerfo, P., Krupey, J. & Hansen, H.J. (1971).
Demonstration of an antigen common to serveral varieties of neoplasia using zirconyl phosphate gel.
New Eng. J. Med. 285, 138-141.

LoGerfo, P. & Herter, F. (1972).
Demonstration of tumor associated antigen in normal colon and lung.
J. Surg. Oncol. 4, 1-7.

LoGerfo, P., Herter, F.P. & Bennett, J.J. (1972).
Absence of circulating antibodies to carcinoembryonic antigen in patients with gastrointestinal malignancies.
Int. J. Cancer 9, 344-348.

LoGerfo, P., LoGerfo, F., Herter, F., Barker, H.G. & Hansen, H.J. (1972a).
Tumor-associated antigen in patients with carcinoma of the colon.
Am. J. Surg. 123, 127-131.

Loor, F., Forni, L. & Pernis, B. (1972).
The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins.
Eur. J. Immunol. 2, 203-212.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951).
Protein measurement with the Folin phenol reagent.
J. Biol. Chem. 193, 265-275.

Lowy, D.R., Rowe, W.P., Teich, N. & Hartley, J.W. (1971).
Murine leukemia virus: High frequency activation *in vitro* by 5-Iododeoxyuridine and 5-bromodeoxyuridine.
Science 174, 155-156.

Mach, J.P. & Pusztaszeri, G. (1972).
Carcinoembryonic antigen: Demonstration of a partial identity between CEA and a normal glycoprotein.
Immunochem. 9, 1031-1034.

Mach, J.P., Pusztaszeri, G., Dysli, M., Kapp, F., De Haan, B., Loosli, R.M., Grub, P. & Isliker, H. (1973).
Dosage radio-immunologique de l'antigene carcino-embryonnaire dans le plasma de malades atteints de carcinomes.
Schweiz. med. Wschr. 103, 365-371.

Mach, J.P., Jaeger, P.H., Bertholet, M.M., Ruegsegger, C.A., Loosli, R.M. & Pettavel, J. (1974).
Detection of recurrence of large-bowel carcinoma by radioimmunoassay of circulating carcinoembryonic antigen.
Lancet ii, 535-540.

MacKay, A.M., Patel, S., Carter, S., Stevens, U., Laurence, D.J.R., Cooper, E.H. & Neville, A.M. (1974).
Role of serial plasma CEA assays in detection of recurrent and metastatic colo-rectal carcinomas.
Brit. Med. J. iv, 382-385.

MacSween, J.M. (1975).
The antigenicity of carcinoembryonic antigen in men.
Int. J. Cancer 15, 246-252.

Markert, C.L. (1968).
Neoplasia: A disease of cell differentiation.
Cancer Res. 28, 1908-1914.

Martin, F., Martin, M.S., Bordes, M. & Bourgeaux, C. (1972).
The specificity of carcinofetal antigens of human digestive tract tumors.
Eur. J. Cancer 8, 315-321.

Martin, F. & Devant, J. (1973).
Carcinoembryonic antigen in normal human saliva.
J. Nat. Cancer Inst. 50, 1375-1379.

Masseyeff, R. (1972).
Human alpha-fetoprotein.
Pathol. Biol. Semaine Hosp. 20, 703-725.

McConahy, P.J. & Dixon, F.J.A. (1966).
A method of trace iodination of proteins for immunologic studies.
Intern. Arch. Allergy Appl. Immunol. 29, 185-189.

Miller, A.B. (1974).
The joint National Cancer Institute of Canada/ American Cancer Society study of a test for carcinoembryonic antigen.
Cancer 34, 932-935.

Moore, T.L., Kupchik, H.Z., Marcon, N. & Zamcheck, N. (1971)
Carcinoembryonic antigen assay in cancer of the colon and pancreas and other digestive tract disorders.
Amer. J. dig. Dis. 16, 1-7.

Moore, T., Dhar, P., Zamcheck, W., Keeley, A., Gottlieb, L. & Kupchik, H.Z. (1971a).
Carcinoembryonic antigen(s) in liver disease.
Gastroenterology 63, 88-101.

Murphy, E.A. (1972).
The normal, and the perils of sylleptic argument.
Perspect. Biol. Med. 15, 566-582.

Neville, A.M. & Laurence, D.J.R. (1974).
Report of the workshop on the carcinoembryonic antigen.
Int. J. Cancer 14, 1-18.

Nowell, P.C. (1976).
The clonal evolution of tumor cell populations.
Science 194, 23-28.

Parks, W.P. & Scolnick, E.M. (1973).
Murine mammary tumor cell clones with varying degrees of virus expression.
Virol. 55, 163-173.

Parks, W.P., Scolnick, E.M. & Kozikowski, E.H. (1974).
Dexamethasome stimulation of murine mammary tumor virus expression.
Science 184, 158-160.

Pierce, G.B. (1970).
Differentiation of normal and malignant cells.
Fed. Proc. 29, 1248-1254.

Plow, E.F. & Edgington, T.S. (1975).
Isolation and characterization of a homogeneous isomeric species of carcinoembryonic antigen: CEA-5.
Int. J. Cancer 15, 748-761.

Prehn, R.T. (1976).
Tumor progression and homeostasis.
Adv. Cancer Res. 23, 203-236.

Pusztaszeri, G. & Mach, J.P. (1973).
Carcinoembryonic antigen in non-digestive cancerous and normal tissues.
Immunochem. 10, 197-204.

Rabellino, E., Colon, S., Grey, H.M. & Unanue, E.R. (1971).
Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation.
J. Exp. Med. 133, 156-167.

Rebhurn, L.I. (1977).
Cyclic nucleotides, calcium and cell division.
Int. Rev. Cytol. 49, 1-54.

Reynoso, G., Chu, T.M., Holyoke, D., Cohen, E., Valensuela, L.A., Nemoto, T., Wang, J.J., Chunag, J., Guinan, P. & Murphy, G.P. (1972).
Carcinoembryonic antigen in patients with different cancers.
J. Am. Med. Assoc. 220, 361-365.

Robinson, G.A., Butcher, R.W. & Sutherland, E.W. (1971).
Cyclic AMP.
Academic Press, New York/London.

Rogers, G.T. (1976).
Heterogeneity of carcinoembryonic antigen: Implications on its role as a tumor marker substance.
Biochim. Biophys. Acta 458, 355-373.

Rosai, J., Tillack, T.W. & Marchesi, V.T. (1972).
Membrane antigens of human colonic carcinoma and non-tumoral colonic mucosa: Results obtained with a new isolation method.
Int. J. Cancer 10, 357-367.

Rosenthal, K.L., Palmer, J.L., Harris, J.A., Rawls, W.E. & Tompkins, W.A.F. (1975).
Antibody-induced redistribution of CEA on the cell surface: Utilization in separation of CEA and isoantigen A.
J. Immunol. 115, 1049-1053.

Rosenthal, K.L., Tompkins, W.A.F., Frank, G.L., McCulloch, P. & Rawls, W.E. (1977).
Variants of a human colon adenocarcinoma cell line which differ in morphology and carcinoembryonic antigen production.
Cancer Res. 37, 4024-4030.

Rule, A.H., Straus, E., van der Voorde, J., & Janowitz, H.D. (1972).
Tumor-associated (CEA-reacting) antigen in patients with inflammatory bowel disease.
New Eng. J. Med. 287, 24-26.

Rule, A.H. (1973).
Carcinoembryonic antigen: Activity of meconium and normal colon extracts.
Immunol. Comm. 2, 15-24.

Rule, A.H. & Goleski-Reilly, C. (1973).
Carcinoembryonic antigen "fingerprints".
Brit. J. Cancer 28, 464-468.

Rule, A.H. & Goleski-Reilly, C. (1974).
Phase-specific oncocolon antigens: A theoretical framework for "carcinoembryonic antigen" specificities.
Cancer Res. 34, 2083-2087.

Rule, A.H. & Kirch, M.E. (1976).
Gene activation of molecules with carcinoembryonic antigen determinants in fetal development and in adenocarcinoma of the colon.
Cancer Res. 36, 3503-3509.

Ruoslahti, E., Pihko, H. & Seppala, M. (1974).
Alpha-fetoprotein: Immunochemical purification and chemical properties.
Transpl. Rev. 20, 38-60.

Sackett, D.L. (1973).
The usefulness of laboratory tests in health-screening programs.
Cl. Chem. 19, 366-372.

Sackett, D.L. (1975).
Laboratory screening: A critique.
Fed. Proc. 34, 2157-2161.

Schöne, G. (1906).
Untersuchungen uber Kakzinomimmunitat bei mausen.
Muench. Med. Wochschr. 53, 2517-2519.

Schreiner, G.F. & Unanue, E.R. (1977).
Capping and the lymphocyte: Models for membrane reorganization.
J. Immunol. 119, 1549-1551.

Scolnick, E.M., Young, H.A. & Parks, W.P. (1976).
Biochemical and physiological mechanisms in glucocorticoid hormone induction of mouse mammary tumor virus.
Virol. 69, 148-156.

Sell, S., Becker, F.F., Leffert, H.L. & Watabe, H. (1976).
Expression of an oncodevelopmental gene product during fetal development and adult oncogenesis.
Cancer Res. 36, 4239-4249.

Silagi, S. & Bruce, S.A. (1970).
Suppression of malignancy and differentiation in melanotic melanoma cells.
Proc. Natl. Acad. Sci. USA 66, 72-78.

Simmons, D.A.R. & Perlman, P. (1973).
CEA and blood group substances.
Cancer Res. 33, 313-322.

Singer, S.J. & Nicolson, G.L. (1972).
The fluid mosaic model of the structure of cell membranes.
Science 175, 720-731.

Singer, R.M., Tompkins, W.A.F., White, L.J. & Perry, J.E. (1976).
Coproduction of Regan isoenzyme and carcinoembryonic antigen in HCT-8 cells.
J. Nat. Cancer Inst. 56, 175-178.

Skarin, A.T., Delwiche, R., Zamcheck, N., Lokich, J.J. & Frei, E. (1974).
Carcinoembryonic antigen: Clinical correlation with chemotherapy for metastatic gastrointestinal cancer.
Cancer 33, 1239-1245.

Skvtelsky, E. & Hardy, B. (1976).
Regeheration of plasmalema and surface properties in macrophages.
Exp. Cell. Res. 101, 337-345.

Spiro, R.G. (1970).
Glycoproteins.
Ann. Rev. Biochem. 39, 599-638.

Steele, G. & Sjugren, H.O. (1974).
Embryonic antigens associated with chemically-induced colon carcinomas in rats.
Int. J. Cancer 14, 435-444.

Steinberg, M.L. & Whittaker, J.R. (1976).
Stimulation in a melanoma cell line by theophylline.
J. Cell Physiol. 87, 265-276.

Straus, E., Vernace, S., Janowitz, H. & Paronetto, F. (1975).
Migration of peripheral leukocytes in the presence of carcinoembryonic antigen.
Proc. Soc. Exp. Biol. Med. 148, 494-497.

Sugarbaker, P.H., Zamcheck, N. & Moore, F.D. (1976).
Assessment of serial carcinoembryonic antigen assays in postoperative detection of recurrent colorectal cancer.
Cancer 38, 2310-2315.

Takeuchi, Y.K. & Kajishima, T. (1976).
Inhibitory effects of dibutyryl cyclic AMP and theophylline on the melanosome transformation in the embryonic chick pigmented retina cultured *in vitro*.
Dev. Biol. 53, 178-189.

Tartarinov, Y.S. (1964).
Content of embryo specific alpha-globulin in blood serum of human fetus, newborn and adult man in primary cancer of liver.
Vopr. Med. Khim. 10, 90-94.

Taylor, R.B., Duffus, W.P.H., Raff, N.C. & de Petris, S. (1971).
Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody.
Nature New Biol. 233, 225-229.

Terry, W.P., Henkart, P.A., Coligan, J.E. & Todd, C.W. (1972).
Structural studies of the major glycoprotein in preparations with carcinoembryonic antigen activity.
J. Exp. Med. 136, 200-204.

Terry, W.D., Henkart, P.A., Coligan, J.E. & Todd, C.W. (1974).
Carcinoembryonic antigen: Characterization and clinical applications.
Transpl. Rev. 20, 100-129.

Thomson, D.M.P., Krupey, J., Freedman, S.O. & Gold, P. (1969).
The radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system.
Proc. Nat. Acad. Sci. USA 64, 161-167.

Tompkins, W.A.F., Watrach, A.M., Schmale, J.D., Schultz, R. & Harris, J.A. (1974).
Cultural and antigenic properties of newly established cell strains derived from adenocarcinomas of the human colon and rectum.
J. Nat. Cancer Inst. 52, 1101-1110.

Tuberville, C., Darcy, D.A., Laurence, D.J.R., Jones, E.W. & Neville, A.M. (1973).
Studies on carcinoembryonic antigen and a related glycoprotein.
Immunochem. 10, 841-843.

Turner, M.D., Olivares, T.A., Harwell, L. & Kleinman, M.S. (1972).
Further purification of perchlorate-soluble antigens from human colonic carcinomata.
J. Immunol. 108, 1328-1339.

Unanue, E.R. & Karnovsky, M.J. (1974).
Ligand-induced movement of lymphocyte membrane macromolecules.
J. Exp. Med. 140, 1207-1220.

Uriel, J. (1969).
Transitory liver antigens and primary hepatoma in man and rat.
Path. Biol. Semaine Hosp. 17, 877-884.

Uriel, J. (1975).
Fetal characteristics of cancer.
IN: *Cancer* Vol. 3 (F.F: Becker, ed.) Plenum Press, New York. pp. 21-56.

von Kleist, S. & Burtin, P. (1969).
Localisation cellulaire d'un antigene embryonnaire de tumeurs coliques humaines.
Int. J. Cancer 4, 874-879.

von Kleist, S., Chavanel, G. & Burtin, P. (1972).
Identification of an antigen from normal human tissue that cross-reacts with the carcinoembryonic antigen.
Proc. Nat. Acad. Sci. USA 69, 2492-2494.

Vrba, R., Alpert, E. & Isselbacher, K.J. (1975).
Carcinoembryonic antigen: Evidence for multiple antigenic determinants and isoantigens.
Proc. Nat. Acad. Sci. USA 72, 4602-4606.

Wepsic, H.T. & Sell, J. (1974).
Alpha-fetoprotein: Expression in human disease and in rat experimental models.
Progr. exp. Tumor Res. 19, 297-324.

Westwood, H.H., Bessell, E.M., Bukhari, M.A., Thomas, P. & Walker, J.M. (1974).
Studies on the carcinoembryonic antigen.
Immunochem. 11, 811-818.

Westwood, J.H. & Thomas, P. (1975).
Studies on the structure and immunological activity of carcinoembryonic antigen - The role of disulphide bonds.
Brit. J. Cancer 32, 708-719.

Wilson, J.D., Nossal, G.J.V. & Lewis, H. (1972).
Metabolic characteristics of lymphocyte surface immunoglobulins.
Eur. J. Immunol. 2, 225-232.

Witebsky, E. (1930).
Sur Serologischen Spezifitat des Karzinomgewebes.
Klin. Wochschr. 9, 58-63.

Yahari, I. & Edelman, G.M. (1972).
Restriction of mobility of lymphocyte immunoglobulin receptors by Concanavalin A.
Proc. Nat. Acad. Sci. USA 69, 608-612.

Zamcheck, N., Moore, T.L., Dhar, P., Kupchik, H.Z. & Sorokin, J.J. (1972).
Carcinoembryonic antigen in benign and malignant disease of the digestive tract.
NCI Monogr. 35, 433-439.

Zamcheck, N., Moore, T.L., Dhar, P. & Kupchik, H. (1972a).
Immunologic diagnosis and prognosis of human digestive tract cancer: Carcinoembryonic antigens.
New Eng. J. Med. 286, 83-86.

Zamcheck, N. (1975).
The present status of CEA in diagnosis, prognosis and evaluation of therapy.
Cancer 36, 2460-2468.

Zamcheck, N., Kupchik, H.Z. & Pusztaszeri, G. (1975).
CEA-5: A more specific CEA?
New Eng. J. Med. 293, 145-146.

Zamcheck, N. (1976).
The present status of carcinoembryonic antigen in diagnosis, detection of recurrence, prognosis and evaluation of therapy of colonic and pancreatic cancer.
Clin. Gastroent. 5, 625-638.