IMMUNOSTIMULATORY PROPERTIES OF CARRAGEENANS

By

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ABSTRACT

The capacity of carrageenan to enhance the primary humoral immune response to foreign erythrocytes was assessed using the Jerne plaque assay. It was found that kappa-carrageenan had an adjuvant effect for both the IgM and IgG response of BALB/c mice to sheep red blood cells. This adjuvant effect was dependent on the dose of carrageenan administered, the genetic background of the experimental animals (BALB/c, C57BL/6, and BALB/c x C57BL/6 F_1 hybrid mice were used), the time of carrageenan injection (relative to antigen), and the type of carrageenan used (kappa-, iota-, and lambda-carrageenans were used).

The direct addition of kappa-carrageenan to cultures of spleen cells also had an adjuvant effect for the anti-SRBC response. This *in vitro* effect depended on the dose of carrageenan added, the genetic background of the spleen cell donors, and the type of carrageenan used.

In order to assess the mode of action of carrageenan's (*in vivo*) immunostimulatory activity, the capacity of spleen cells from mice which had been injected with carrageenan to modulate an *in vitro* immune response was studied. It was found that carrageenan-treated cells

released a soluble factor(s) which enhanced the *in vitro* anti-sheep red blood cell response of murine spleen cells. The amount of enhancement observed *in vitro* was directly correlated to the dose of carrageenan given *in vivo* suggesting that inducing the release of soluble enhancing factor(s) was the basis for carrageenan's adjuvant effect.

Cell fractionation experiments revealed that the soluble enhancing factor(s) was produced by a non-adherent T cell and that cell-cell interactions were involved in its production. The possibility that the soluble enhancing factor was interleukin 2 was discussed.

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INTRODUCTION

This thesis describes some effects of carrageenans on the immune response with emphasis on the immunostimulatory properties of these molecules. Consequently, I will begin this discussion by characterizing the immune response. Then I will describe some of the molecules which have the capacity to stimulate the immune response. Finally I will describe carrageenans and the effects these molecules have on the immune response.

1.1 The Immune Response

An immune response is a biological reaction to a substance which has been recognized as foreign. The purpose of this response is to protect the organism from pathogenic agents. The abilities to recognize "foreigness" and to acquire "memory" are two important features of the immune system. The immune response is dependent on specialized cells (lymphocytes) and cell-cell interactions.

A substance which causes an immune response is called an antigen. The site(s) on an antigen which is recognized by

the immune system is known as the antigenic determinant and a molecule must have at least two of these to be immunogenic. Haptens are molecules which possess only one antigenic determinant. A hapten can become immunogenic if it is chemically coupled to an antigen. Specific immune responses are initiated by the hapten and the carrier to which it is coupled. The products of the immune response are extremely specific and can discriminate between antigens which show very little structural difference. For example, it has been demonstrated that an immune response directed towards L-tartaric acid will not react with D-tartaric acid (Landsteiner, 1945). This specificity is important since many foreign molecules are structurally similar to self components and a lack-of discrimination in antigen recognition could result in autoimmune reactions.

The ability of the immune system to respond more vigorously to a second exposure to a given antigen is called immunologic memory. This ability of the immune system enhances its protective capacity. Because of immunologic memory vaccination is an effective prophylactic technique.

Immune responses may be classified as being either cell-mediated or humoral in nature. Cell-mediated responses are typically elicited by parasites, fungi, intracellular viral infections, and foreign or neoplastic cells, whereas humoral immune responses are initiated by extracellular

bacterial and viral infections. Typical cell-mediated reactions include: delayed hypersensitivity (Dvorak et al., 1976), allograft rejection (Lafferty and Woolnough, 1977), and graft-versus-host disease (Grebe and Streilin, 1976). In vitro correlates of cell-mediated immunity are the mixed-lymphocyte reaction (Dupont et al., 1976) and cell-mediated lympholysis (Berke, 1980). This thesis concerns itself with the humoral immune response and cell-mediated reactions will not be discussed further. The principles previously described apply equally to cell-mediated and humoral immune responses.

1.1.1 Antibody Structure

The effector molecules of humoral immune responses are antibodies. Antibodies are antigen-specific molecules which belong to a group of serum proteins called immunoglobulins. Immunoglobulins (Ig) are differentiated into five classes: IgG, IgM, IgA, IgD, and IgE.

A typical monomeric immunoglobulin molecule consists of four disulfide bond-linked polypeptide chains: two heavy (high molecular weight) chains and two light (low molecular weight) chains. The amino terminal portion of the light and heavy chains shows considerable variability in amino acid sequence and is known as the variable region.

The majority of the heavy chain and the carboxy terminal half of the light chain show little amino acid sequence variability among immunoglobulins of a given class and together are referred to as the constant region. The variable regions of the heavy and light chains form the antigen-binding site of the antibody molecule. There are two of these sites on a monomeric antibody molecule. The constant portion of the heavy chain determines most of the effector functions of the antibody such as cytotropism and complement fixation. Each antibody molecule exhibits a unique structure in its variable region due to the specificity of a particular antibody for a given antigen. This structure is called the idiotypic determinant of the antibody. Antibody structure has been recently reviewed (Hood et al., 1975; Metzger, 1979), antibody effector function has been discussed by Winkelhake (1978), and idiotypes are described by Capra (1977).

1.1.2 Cellular Basis for Antibody Production

Antibodies are produced by a class of lymphocytes known as B cells (originally bone-marrow derived lymphocytes). Each B cell produces antibody of a unique structure and is capable of being triggered into clonal expansion and antibody production only by the appropriate

antigen(s). This triggering of B cells to produce antibody requires, in addition to antigen, T cells (thymus-derived lymphocytes) (Waldmann, 1977; Russell and Tomasi, 1978; Cantor, 1979; Campos-Neto et al., 1978; Katz and Benacerraf, 1972; Herzenberg et al., 1976) and accessory cells (Miller, 1979; Feldmann et al., 1979; Unanue, 1978; Gorczynski, 1977) for most if not all antigens. The usefulness of this multicellular requirement is that it facilitates the control of immune responses.

B cells and T cells are lymphocytes which are morphologically indistinguishable from each other. They both arise from the same stem cell in the bone marrow but then their developmental pathways diverge (Abramson et al., 1977). Pre-T cells migrate to the thymus gland where they differentiate and proliferate into mature T cells prior to seeding the peripheral lymphoid organs and the circulation (Naylor and Goldstein, 1979). Pre-B cells migrate to the fetal liver in mammals (Owen et al., 1974; Lawton and Cooper, 1978; Melchers et al., 1975) or to the bursa of Fabricius in birds (Melchers et al., 1975; Lawton et al., 1975) where they mature before moving into the lymphoid tissues and circulation. In the adult mammal B cells arise directly from the bone marrow and spleen (Lawton and Cooper, 1978; Melchers et al., 1975). There is some controversy concerning the requirement for an intact thymus gland for

T-cell maturation. Congenitally athymic mice (nude mice) have been shown to have cells with T-cell markers (Raff, 1973; Roelants et al., 1976) and functionally active T cells have been grown in vitro from cultures of nude mouse spleens by Jacobs and Miller (1979). It may be that in the absence of a thymus some other organ can take over the function of facilitating T-cell development.

T cells and B cells can be differentiated from each other by the presence of different cell surface molecules. Antibody directed against these molecules can be used to label or kill selected populations of cells.

The most important cell surface marker for identifying murine T cells is the thy 1 antigen (Reif and Allen, 1964). It is acquired during passage through the thymus gland and is not found on pre-T cells. The thy 1 antigen is not found on B cells but it is not restricted to T cells. The Lyt (Ly = lymphocyte, t = T cell) antigens are a group of differentiation antigens found exclusively on T cells. Other Ly antigens are found on different cells such as LyB antigens on B cells. Cantor and Boyse (1975) characterized two loci in this group of antigens; Lyt-1 and Lyt-2/3. T cells may be classified as follows with anti-Lyt antisera: Lyt $1^+2^+3^+$ cells appear first in ontogeny and comprise 50% of the thy 1^+ cells, Ly-1^+ cells constitute 33% of the thy 1^+ cells, and

Lyt-2^+3^+ cells contribute 6% of the thy 1^+ cells (Cantor and Boyse, 1975). The presence of different Lyt antigens delineates functionally distinct T-cell subpopulations (see below).

B cells can be identified by the presence of cell surface immunoglobulin (Jerry and Sullivan, 1976). This immunoglobulin has the same antigen specificity as the antibody which will subsequently be produced by that B cell. The cell surface immunoglobulin is embedded in the cell membrane by the constant portion of the heavy chain with its antigen receptors exposed on the cell surface.

B and T cells can also be differentiated on the basis of their proliferative responses to mitogens. T cells respond mitogenically to the plant lectins phytohemagglutinin and concanavalin A (Stobo et al., 1972) whereas the definitive B cell mitogen is bacterial lipopolysaccharide (Axelrod and Shands, 1977).

Both B and T cells recognize antigen. There is good evidence that the B-cell antigen receptor is cell surface immunoglobulin (Jerry and Sullivan, 1976; Katz and Benacerraf, 1972). The nature of the T-cell antigen receptor, however, is still unclear. Many attempts have been made to demonstrate the presence of cell surface immunoglobulin on T cells. Anti-immunoglobulin antisera blocked antigen binding by T cells but this was not

consistently observed (Jerry and Sullivan, 1976; Playfair, 1974). It was suggested that T cells express immunoglobulin light chains coupled to a unique class of heavy chain forming a new immunoglobulin molecule called IgX or IgT (Katz and Benacerraf, 1972). More recent studies suggest that T cells do not bear endogenously synthesized immunoglobulin of any of the known classes, nor do they have identifiable light chains (Paul and Benacerraf, 1977). However, Storb (1978) has shown, using in situ hybridization with DNA complimentary to light chain messenger RNA, the presence of light chain messenger RNA in T cells. It would be surprising if the immunoglobulin light chain was not being made by these T cells.

T cells do bear idiotypic determinants similar to those expressed on the heavy chains of immunoglobulin (Paul and Benacerraf, 1977). This has been shown to occur in the mouse by Julius et al., (1977) and Cosenza et al. (1977), and in the rabbit by Cazenave, et al. (1977). BALB/c mice display a restricted heterogeneity in their antibody response to phosphorylcholine. Antigen-specific anti-idiotype antisera can inhibit the binding of phosphorylcholine to antigen-activated T cells in this strain of mice (Julius et al., 1977; Cosenza et al., 1977). The target of the anti-idiotype antibody was not a molecule passively absorbed onto the T-cell surface suggesting it

was the functional receptor on phosphorylcholine-specific T cells. T cells have also been prevented from binding antigen by antisera directed towards the K- or D-end products of the H-2 complex (Lonai, et al., 1978) (see below for a discussion of H-2). Taken together these results suggest that the T-cell antigen receptor comprises at least part of the variable portion of the heavy chain and a gene product(s) of the H-2 complex.

The major histocompatibility complex in mice is known as H-2. The genes in this complex code for a variety of proteins involved in immune responses. The H-2 complex is divided into four regions: K, I, S, and D. The K and D regions define cell surface antigens that are involved in the recognition of cells by the immune system. The S locus codes for a component of the complement system. The I region can be divided into subregions denoted: I-A, I-B, I-C, I-E, and I-J. This region codes for a series of cell surface proteins known as Ia antigens. In addition to Ia antigens the I region also contains the immune response genes. These genes determine the capacity of a given individual to respond to antigen and are important in cellular interactions (Benacerraf and Katz, 1975). The product of immune response genes may be Ia antigens (Thomas et al., 1977). The H-2 complex is described in more detail by Bach (1978).

The B-cell's function in humoral immune responses is to produce antibody. The T cell functions to regulate antibody production. The third cell required for antibody production is the accessory cell (such as the macrophage).

Macrophages are morphologically easily distinguished from B and T cells. They are large, irregularly-shaped cells with a large amount of vacuolated cytoplasm. Macrophages arise in the bone marrow from the same primordial stem cell as B and T cells (Abramson et al., 1977). The initial stages of macrophage development occur in the bone marrow where the stem cell differentiates into a myeloid stem cell which in turn becomes a promonocyte (Van Furth, 1970). The promonocyte develops into a monocyte which leaves the bone marrow and enters the circulation. When monocytes leave the blood and enter the tissues they become macrophages (Van Furth, 1970). Macrophages are scattered throughout the tissues of the body being particularly prevalent in the lymphoid organs, the liver, the lungs, and the peritoneal cavity.

Macrophages are distinguished from B and T cells by: their morphology, their capacity for phagocytosis, and their ability to adhere to glass and plastic.

Macrophages bind nonspecifically to microorganisms (Weir and Ogmundsdottir, 1977) but they also have the ability to bind specific antigen. Cohen et al. (1973)

showed that macrophages from immune, but not nonimmune, animals could bind low concentrations of antigen. Both immune and nonimmune macrophages bound high concentrations of antigen. They suggested that the binding of low doses of antigen was due to the presence of an antigen-specific cytophillic antibody on the macrophage surface, whereas the binding of high doses of antigen was caused by a low avidity antigen receptor. Gorczynski et al. (1979) found that carbohydrates differing in their intrachain glucosidic links were bound by different subpopulations (fractionated on the basis of size) of peritoneal macrophages. It has also been shown that macrophages can exercise determinant selection in the immune response to insulin (Rosenthal et al., 1977). Taken together these results indicate that macrophages may have more antigen specificity than previously thought. Although the recognition of antigen by macrophages is probably less specific than antigen recognition by T cells and B cells, macrophages may be segregated into overlapping populations which are capable of recognizing different types of antigenic determinants. Cytophillic antibody, bound to the macrophage surface, gives these cells the antigen discriminating ability of B cells (provided antibody specific for only one antigen is bound to them).

Macrophages function in the humoral immune response

as processors and presenters of antigen. In addition, these cells seem to be able to regulate T- and B-cell function thus giving the immune response a third level of control. Macrophages, B cells, and T cells are compared in Table 1.

1.1.3 Activation of B cells

One of the major questions in the field of immunology is "how are B cells activated to produce antibody?" Table 2 outlines the principal tenets of the one-signal and two-signal models proposed to explain B-cell activation.

Neither model appears to be able to explain B-cell activation to all antigens. Coutinho and Moller (1974) have argued that because mitogens can cause the polyclonal activation of B cells to antibody production, the two-signal model is incorrect. This phenomenon suggests that B cells may be activated by some signal unrelated to their antibody receptors and the two-signal theory that delivery of signal two alone can lead to activation of B cells is incompatible with self tolerance. Recent evidence (Bretscher, 1978) has suggested that the activation of B cells by lipopolysaccharide is related to endogenous antigenic stimulation. If this can be convincingly shown

TABLE 1. A COMPARISON OF THE CELLS REQUIRED FOR MURINE HUMORAL IMMUNE RESPONSES

Cell Type	Morphology	Surface Markers	Mitogens	Function
B cell	small to medium in size, with a round nucleus and little cytoplasm	immunoglobulin	lipopolysaccharide	antibody production
T cell	same as B cells	thy 1 antigen, Lyt antigens	phytohemagglutinin, concanavalin A	regulation of B-cell activity
Macrophage	large cells with a kidney-shaped nucleus and a large amount of cytoplasm	macrophage-specific antigens	none	antigen processing and presentation, regulation of B-cell and T-cell activity

TABLE 2. A COMPARISON OF TWO MODELS FOR EXPLAINING B-CELL ACTIVATION

ame of Model	Reference	Function of Cell-Surface Immunoglobulin	Response to T-cell Dependent Antigen	Response to T-cell Independent Antigen	Tolerance Due To	Polyclonal Activation Due To
ne-signal	Coutinho and Moller, 1974	Passive, focuses antigen on appropriate B-cell but receives no signal	The activating signal is delivered to the B-cell receptor (mitogen receptor) by an antigen-specific T-cell or T-cell factor via antigen bridging	T-independent antigens exhibit intrinsic mitogenicity which can activate B-cells (via mitogen receptors) without T-cells	Delivery of too much signal	Delivery of signal without antigen specificity
Two-signals	Cohn and Blomberg, 1975	Receives signal one from antigen	The activating signal (signal two) is delivered by the T-cell system	T-independent antigens do not exist but are antigens which require less T-cell signal	Delivery of signal one only	Delivery of signal two alone (mitogenic stimulus) leads to low levels of activation

then polyclonal activation of B cells could be explained by a two-signal model. It may be, as Marshall-Clark and Playfair (1979) have suggested, that B cells can be divided into distinct subsets which have different triggering requirements and therefore both theories are essentially correct but apply to different B-cell subpopulations.

1.1.4 Control of B-Cell Activation

A potent source of second signals for triggering B cells is the helper T cell. These cells belong to the Lyt 1^+ subclass of the thy 1^+ cells (Cantor et al., 1976). Feldmann et al. (1977) showed that the generation of helper function by Ly 1^+ T cells required the participation of Lyt $1^+2^+3^+$ T cells. They found that the latter population functioned as amplifier cells. Lyt $1^+2^+3^+$ T cells can also inhibit Lyt 1^+ T-cell activity after receiving appropriate signals from Lyt 1^+ cells (Cantor, 1979). Thus, Lyt $1^+2^+3^+$ T cells are intimately involved in the regulation of helper T cells both in a stimulatory and inhibitory sense. It may be that these two activities are associated with discrete subpopulations of Lyt $1^+2^+3^+$ cells.

T and B cells must be histocompatible for optimal cell interactions to occur (Waldmann, 1977; Paul and

Benacerraf, 1977; Benacerraf and Katz, 1975). It has been suggested that this genetic restriction does not apply to unprimed T and B cells if allogeneic effects are removed (Paul and Benacerraf, 1977) and in fact T cells can release soluble factors which can enhance the antibody response of histoincompatible B cells (Feldmann and Basten, 1972). Primed T and B cells must be similar in the I-A subregion of the H-2 complex in order to elicit a secondary antibody response (Paul and Benacerraf, 1977). Paul and Benacerraf (1977) suggested that, since T cells responded to a secondary stimulus only with the B cells they were primed with, antigen may be recognized by the T cell in the context of an appropriate major histocompatability complex gene product made by the B cell.

The second signal, provided by helper T cells, which activates B cells that have reacted with antigen, is probably a soluble factor(s). Two types have been defined, antigen-specific factors and antigen-non-specific factors.

T cells, cultured in the absence of fetal calf serum, produced a soluble factor which stimulated the in vitro immune response in T-cell depleted spleen cell cultures to specific antigen (Watson, 1973). The release of this stimulatory factor was enhanced by the presence of

specific antigen [sheep red blood cells (SRBC)]. Mozes and Haimovich (1979) were able to produce an antigen-specific T-cell factor by incubating T-cells with poly (Tyr,Glu)-poly(DLAla)--poly(Lys) [(T,G)-A--L]. This factor, which replaced the need for T cells in the antibody response to (T,G)-A--L, was found to possess idiotypic determinants similar to those found on anti-(T,G)-A--L antibodies (Mozes and Haimovich, 1979). Schreier and Tees (1980) showed that antigen-specific T cells, in the presence of specific antigen and macrophages, released a soluble factor that stimulated antibody production by B cells. This soluble factor would only act on B cells in the presence of antigen but the antigen could be different from the one which stimulated the T cells.

Feldmann and Basten (1972) reported that T cells, activated by allogeneic interaction, released a low molecular weight soluble factor which increased the antibody response of nude mouse spleen cells in vitro. This soluble factor stimulated the immune response to a variety of antigens including T-cell dependent and T-cell independent antigens. The admixture of mouse spleen cells with concanavalin A can generate supernatants which stimulate antibody production by B cells (Watson et al., 1979a; Bernabe et al., 1979). Watson et al. (1979b) found that the active factor had a molecular weight of

30,000 daltons and consisted of two components with identical biological properties. They identified this molecule as T-cell growth factor and among its properties found it generated antibody-forming cells in T-cell depleted cultures. Bernabe et al. (1979) found that concanavalin A-generated supernatants were polyclonal mixtures of helper activities with diverse specificities. Specificity could be demonstrated by enrichment or depletion of antigen-reactive cells in the T-cell-concanavalin A mixture.

Thus, it seems that helper T cells, at least in vitro, can mediate enhancement of antibody production by the release of soluble factors. Some of these factors appear to be antigen-specific in terms of the T cell producing them, but seem to be nonspecific when polyclonally induced by either concanavalin A or an allogeneic reaction. Other factors, such as interleukins, are clearly nonspecific. At least in some cases the T-cell factor may stimulate any B cell which has reacted with antigen.

T cells can also affect the immune response in an inhibitory way. The T cells which accomplish this are known as suppressor T cells. Suppressor T cells belong to the Lyt 2^+3^+ subclass of thy 1^+ cells (Cantor et al., 1976) and express cell surface Ia antigens (Vadas et al.,

1976).

The generation of suppressor T cells that regulate the antibody response requires cellular interactions (Gershon, 1975; Eardley et al., 1979; Feldmann et al., 1977; Eardley and Gershon, 1975). Eardley and Gershon (1975) found that carrier-primed T cells suppressed the response of nonimmune T cells in the intact host. Either population of cells, by itself, could provide helper activity. They suggested that the immune T cells generated suppressor T cells in the host because the host cells were affected by regulatory signals differently from the injected immune T cells. Gershon (1975) speculated that when immune activity is high more feedback products are generated and this preferentially stimulates suppressor T cells. Suppressor T-cell precursors (Lyt 2^+3^+,Ia^-) were found to require an amplifier cell (Lyt $1^+2^+3^+$,Ia^-) to differentiate into suppressor T-cell effectors (Lyt 2^+3^+,Ia^+) (Feldmann et al., 1977). Eardley et al. (1979) suggested that the amplifier cell was stimulated by Lyt 1^+ cells to generate suppressor T cells. In their system the interacting T-cell subpopulations had to be identical at an immunoglobulin gene locus for effective interaction to occur and the authors suggested that cellular interactions required for generating suppressor T cells are governed by immunoglobulin-linked genes.

Thus it seems that helper T cells (Lyt 1^+), suppressor T cells (Lyt 2^+3^+), and Lyt $1^+2^+3^+$ T cells are interlinked in a regulatory network which governs the nature and amount of second signal the B cell will receive.

Suppressor T cells, like helper T cells, probably mediate their function via soluble factors which may also be either antigen-specific or nonspecific. Tada et al. (1975) isolated a soluble suppressor molecule from rats and mice. This molecule, produced by T cells, could suppress an ongoing IgE response and was carrier specific (the suppressor molecule bound to the carrier but not the hapten of the antigenic molecule). The molecule was found to be a protein of molecular weight 35,000-60,000 daltons which did not possess any of the usual immunoglobulin determinants. The suppressor molecule bore antigenic determinants coded for by the K-end of the H-2 complex which the authors felt were most likely I-region gene products. L-glutamic acid ^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)-primed nonresponder mice produced a soluble T-cell factor which suppressed the antibody response to GAT (Theze et al., 1977). This factor was found to be a GAT-binding protein but showed no antibody determinants although it did have I-region determinants. Taussig and Holliman (1979) described an antigen-specific (SRBC) suppressor T-cell factor. The suppressor molecule consisted of two types of

polypeptide chains a heavy one, weighing 85,000 daltons, and a light one with a molecular weight of 25,000 daltons. The heavy chain bound to SRBC while the light chain was removed by anti-H-2 antiserum. The molecule was unaffected by treatment with anti-mouse immunoglobulin.

Soluble immune response suppressor is produced by T cells which have been stimulated by concanavalin A (Russell and Tomasi, 1978). This molecule was not antigen specific and could block both primary and secondary antibody responses. Like the antigen-specific suppressor molecules described above, soluble immune response suppressor did not have immunoglobulin determinants.

Thus T cells also produce a group of suppressor molecules many of which are antigen-specific and possess H-2 determinants. In terms of the two-signal model suppressor T-cell factors may be considered as molecules which prevent the delivery of signal two to the B cell, thereby preventing B-cell activation.

Accessory cells are also involved in the generation of humoral immune responses. These cells interact with B cells and T cells. Macrophage-B-cell interaction is a required step for most antibody responses (Feldmann et al., 1979). Mongini et al. (1978) demonstrated the requirement for macrophages for the activation of B cells by anti-IgM antisera. They speculated that macrophages may contribute

secondary signals needed to push B cells further into the cell cycle. Macrophage-B-cell interaction has been shown to require cell contact but it is unclear whether or not the two cell types must be histocompatible (Feldmann et al., 1979). It has been suggested that macrophage-B-cell interaction involves the presentation of macrophage-modified antigen, or T-cell factors complexed with antigen, to the B cell.

The induction of helper T cells was shown to require the presence of macrophages in vitro (Niederhuber and Allen, 1980; Feldmann et al., 1979). Helper T cells and macrophages must be histocompatible in order for helper T-cell induction to occur and for some antigens the I-A subregion has been identified as the major area for the requirement of syngenicity (Feldmann et al., 1979). For an antibody response to erythrocyte antigens the helper T cells and macrophages must be identical at the I-J subregion of the H-2 complex (Niederhuber and Allen, 1980). Helper T cells possessed genetically restricted receptors for macrophage I-J subregion gene products. The interaction between this receptor and the macrophage I-J subregion determinants was found to be essential for the initiation of a primary in vitro antibody response (Niederhuber and Allen, 1980).

Like T cells, macrophages can effect the immune

response via soluble mediators. The in vitro antibody response of murine spleen cells to a T-cell independent antigen required the presence of macrophages or 24 hr culture supernatants from these cells (Nordin, 1978). Production of the active supernatants did not require antigen and the supernatant-producing cell did not have to be histocompatable with the responding B cell. Gorczynski (1977) showed that macrophages released protein molecules which reconstituted the antibody response of macrophage-depleted syngeneic lymphocytes. A subpopulation of macrophages released a soluble factor which reconstituted the antibody response of syngeneic and allogeneic lymphocytes (Gorczynski, 1977). Calderon et al. (1975) found that macrophages, activated by protease-peptone or by ingestion of latex particles released an antigen-nonspecific factor which enhanced antibody production in vitro.

Macrophage culture supernatants can also stimulate T-cell function (Young and Mclvor, 1980; Erb et al., 1976). Activated macrophages released a soluble factor which stimulated helper T-cell function (Young and Mclvor, 1980). The amount of T-cell stimulation caused by this factor was related to the degree of activation of the macrophage. Macrophages, stimulated by specific antigen, released a factor (genetically related factor) which

bound to antigen and stimulated helper T-cell induction (Erb et al., 1976). Genetically related factor has Ia antigen determinants.

Macrophages, like T cells, may inhibit antibody responses although soluble suppressor molecules produced by macrophages are not well characterized. Culture fluids, generated by peptone-induced macrophages, suppressed antibody responses of spleen cells at high concentrations of macrophage supernatant (Unanue, 1978). The author suggested that this inhibitory activity was due to the expansion of suppressor T cells by the macrophage-produced factor although there are other possibilities (e.g. release of arginase into the medium by macrophages, depleting arginine).

Products of activated lymphocytes can also effect the secretion of lymphocyte modulatory molecules by macrophages (Unanue, 1976). To complicate matters further, soluble factors produced by T cells, for example soluble immune response suppressor (Russell and Tomasi, 1978), may modulate B-cell function via macrophages. Thus a complicated network of mutually interacting T cells and macrophages controls the production of antibody by B cells. A simplified model, showing the various cellular influences on B cells, is shown in figure 1.

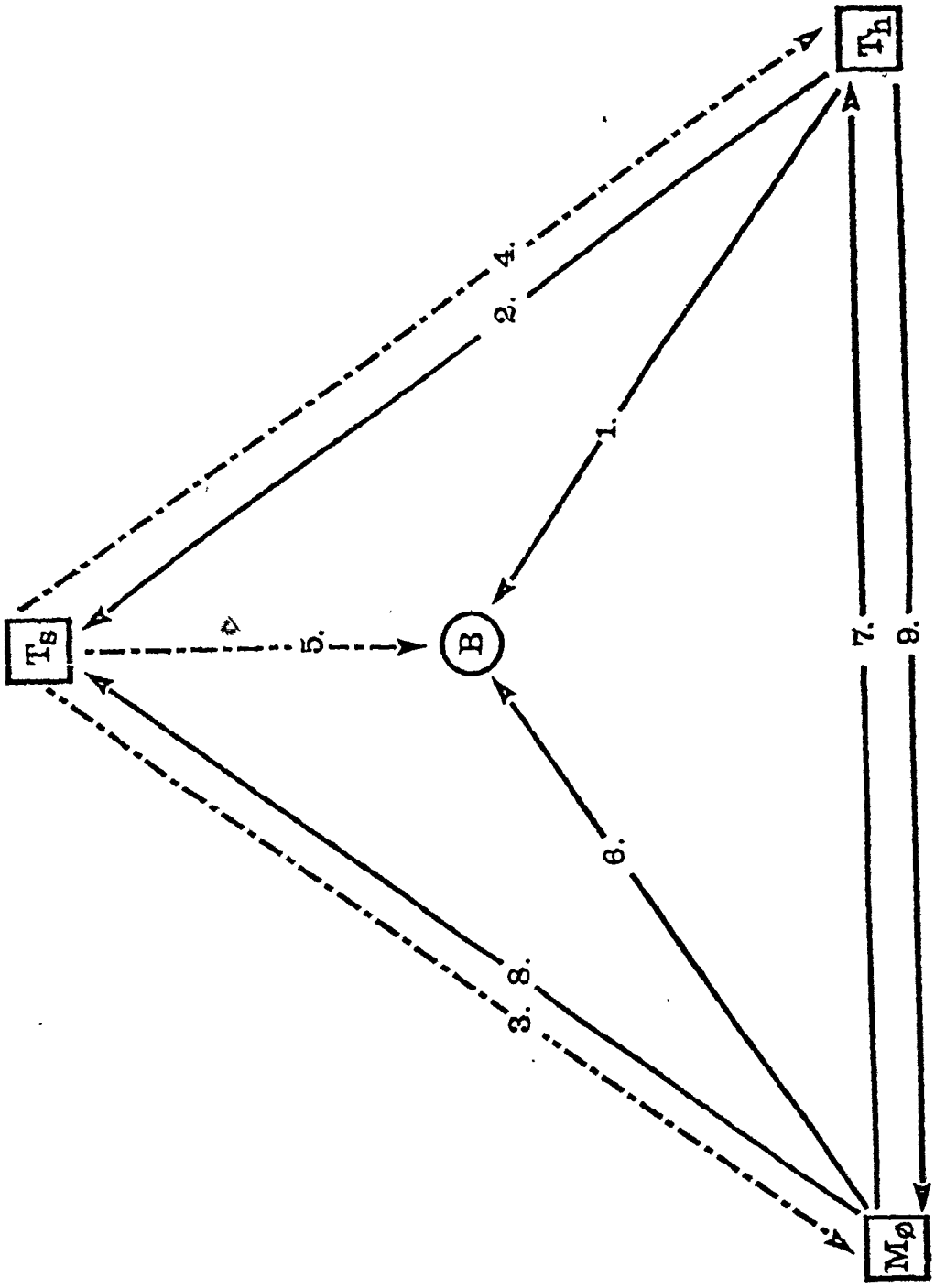
The immune response may also be regulated by

FIGURE 1. CELLULAR REGULATORY FACTORS INFLUENCING B-CELL ACTIVATION.

Legend:
B = B cell
T_H = Helper T cell
Mϕ = Macrophage
Ts = Suppressor T Cell
$\longrightarrow$ = Stimulatory Signal
$\dashrightarrow$ = Inhibitory Signal

The numbers refer to references as follows:

1. Watson, 1973
2. Eardley *et al.*, 1979
3. Russell and Tomasi, 1978
4. Tanssig and Holliman, 1979
5. Tada *et al.*, 1975
6. Nordin, 1969
7. Young and McIvor, 1980
8. Unanue, 1978
9. Unanue, 1971


Tд
Тв
В
Мф
1.
2.
3.
4.
5.
6.
7.
8.

antibody. This regulation may take the form of a network of interacting idiotypes as proposed by Jerne (1974). Recently Herzenberg et al. (1980) have proposed a circuit-based control system which is based on idiotypes but also incorporates the regulatory influence of T cells and macrophages on antibody production.

In summary, the production of antibody by B cells is a highly regulated event. The multiple controls on the immune response serve as mechanisms to avert autoimmune events. In addition, by having an interconnecting web of immunocompetent cells the immune system is more sensitive and responsive to perturbation by antigenic agents.

1.2 Adjuvants

One of the basic aims of research in the field of immunology is to learn how to control the immune response. Substances which can be used to enhance the immune response to a given antigen are known as adjuvants. Table 3 shows a list of substances which have adjuvant properties. In this section I will discuss the effects of adjuvants on the humoral immune response however, it should be noted that adjuvants can also augment cell-mediated responses. As can be seen in the model (figure 1) adjuvants

TABLE 3. A LIST OF IMMUNOLOGIC ADJUVANTS

BACTERIA AND BACTERIAL PRODUCTS:

- *Bordetella pertussis*
- *Brucella abortus*
- Corynebacteria e.g. *C. parvum*
- Lactobacilli
- Mycobacteria e.g. *M. tuberculosis*, *Bacillus Calmette-Guerin*
- Norcardia
- Lipopolysaccharide
- Muramyl dipeptide

LECTINS:

- Concanavalin A
- Phytohemagglutinin

POLYSACCHARIDES:

- Carrageenan
- Dextran sulfate
- Lentinan
- Levamisole

WATER/OIL EMULSIONS:

- Freund's complete and incomplete adjuvants

MISCELLANEOUS COMPOUNDS:

- Alum
- Colchicine
- Glycerol techoic acid
- Lysolecithin analogs
- Nystatin
- Poly adenylic:Poly uradylic acid
- Poly inosinic:Poly cytodylic acid
- Polymethylmethacrylate
- Pyran copolymer
- Retinoic acid
- Silica
- Styrene maleic anhydride

may enhance the immune response by directly affecting B cells or by acting on the cells which regulate the production of antibody. In addition, adjuvants may enhance the humoral immune response by interacting with antigen.

Several adjuvants enhance the immune response by affecting macrophage function (Unanue et al., 1969; Sljivic and Watson, 1977; McGhee et al., 1979a; Fevrier et al., 1978). Unanue et al. (1968) treated macrophages which had bound antigen (hemocyanin) with Bordetella pertussis in vitro. They found that these macrophages could enhance the anti-hemocyanin response of intact recipients whereas macrophages which had only been treated with antigen did not exert an immunostimulatory effect. Similar treatment of lymphocytes, injected into irradiated hosts, showed no adjuvant effect for the anti-hemocyanin immune response. Sljivic and Watson (1977) found that macrophages which were obtained from mice that had been treated in vivo with Corynebacterium parvum, enhanced the in vitro antibody response to SRBC of normal nonadherent spleen cells. McGhee et al. (1979a) looked at the in vitro adjuvant activity of lipopolysaccharide. They found that the addition of lipopolysaccharide to spleen cell cultures together with antigen (SRBC) stimulated the anti-SRBC antibody response. The removal of macrophages from these cultures obliterated the adjuvant

activity of lipopolysaccharide. Muramyl dipeptide can also enhance the *in vitro* immune response of murine spleen cells to SRBC (Fevrier *et al.*, 1978). It was found that supernatants from adherent cells, incubated with muramyl dipeptide, augmented the anti-SRBC response of normal spleen cells (Fevrier *et al.*, 1978). Treatment of the supernatant producing cells with anti-thy 1 plus complement had no effect on the production of enhancing supernatant whereas treatment of these cells with anti-macrophage serum and complement prevented the production of an immunostimulatory supernatant. In the above examples it is difficult to determine exactly how macrophage-adjuvant interaction is causing an enhanced immune response. In the case of Fevrier *et al.*'s experiments (1978) it is clear that the macrophage is releasing a soluble factor (due to adjuvant stimulation) which enhances the immune response, however, it is not clear what the nature of this soluble factor is nor what its cellular target is in the normal spleen cell population.

Adjuvants can also stimulate the immune response via interaction with helper T cells. Bick and Johnson (1977) incubated mouse thymocytes with polyadenylic:polyuridylic acid and looked at the effect of soluble factors released by these cells on the *in vitro* spleen cell

antibody response to burro red blood cells. They found that these soluble factors, released in the absence of antigen, enhanced the antibody response of the spleen cells. Bick and Johnson (1977) suggested that the release of these enhancing soluble factors was due to the polyclonal activation of T cells by polyadenylic:polyuridylic acid. Maillard and Bloom (1972) obtained results similar to Bick and Johnson's using B. pertussis. Adjuvants may also interact with T-cell factors to enhance the immune response. The incubation of B cells, antigen, lipopolysaccharide and a T-cell replacing factor (produced by T cells) gave a synergistically augmented immune response (Jacobs, 1979). The author suggested that lipopolysaccharide induced B cells to be more sensitive to a normally produced T-cell factor. It is also possible that adjuvants could cause expansion of the helper T-cell pool, however because of feedback regulatory loops this would not necessarily result in an increased immune response.

Adjuvants may enhance the immune response by acting directly on B cells. B. pertussis can augment the in vitro anti-SRBC response of normal spleen cells (Murgo and Athanassiades, 1975). It was found that this enhancement could occur in the absence of T cells or macrophages. Because there was no mitogenic stimulus

on the spleen cells by the B. pertussis it would seem that the adjuvant was acting directly on precursors of antibody-forming cells (Murgo and Athanassiades, 1975).

Adjuvants may also act on the antigen to enhance immune responses. Immunostimulants such as alum and ferric oxide cause a slower elimination of antigen and therefore a more prolonged stimulation of the immune response (Borek, 1977). Freund's adjuvants may act, in part, by causing the slow release of antigen from oil-induced granulomas.

It is important to remember that stimulation of the immune response can also result in stimulation of suppressor T cells since they are a normal component of the response. Morris and Johnson (1978) found that the pretreatment of mouse spleen cells with polyadenylic:polyuridylic acid, in vivo or in vitro, caused the induction of suppressor T cells which could inhibit the antibody response of normal spleen cells exposed to antigen.

As can be seen from the above discussion adjuvants have multifaceted activities. For example, B. pertussis can affect macrophage, helper T-cell, and B-cell function. The varied activities of adjuvants and the complexity of the immune response make it very difficult to discern their precise modes of action.

Adjuvants are of great potential use to clinicians.

Immunotherapy has been attempted, using adjuvants, in cases of neoplasia (Milas, 1978; Eilber et al., 1976; Olsson and Ebbesen, 1978) and immunoprophylaxis may also be possible (Sharma, 1977). Adjuvants may be useful in vaccines and could be particularly important in immunity to parasites (Siddiqui et al., 1977). Adjuvants are used in the immunotherapy of allergic diseases and may in the future be used to "turn off" selected components of the immune response since adjuvants can activate suppressor cells (Bennett et al., 1978; Neta and Salvin, 1979; Glaser, 1978; Babcock and McCarthy, 1977). This procedure would be useful in creating autoimmune disorders and for facilitating transplantation. Perhaps the most important use of adjuvants is as an aid for the research immunologist. Adjuvants can be used to promote the induction of animal models of human autoimmune states, to simplify the production of antisera especially of the IgE class (Tada, 1975; Ishizaka, 1976), or to study the role of that cell that a specific adjuvant stimulates in various immunologic phenomena. For these reasons it is important to develop new adjuvants and to analyse their mode of action.

1.3 Carrageenans

1.3.1 Structure

Carrageenans are linear polysaccharides which show no evidence of branching (Anderson et al., 1968b). These molecules are extracted from certain members of the algal division Rhodophyta. Although several types of carrageenan are made, I will limit this discussion to three: kappa-carrageenan, lambda-carrageenan, and iota-carrageenan. These are sulfated polysaccharides with a basic skeleton formed of D-galactose units alternately coupled α 1→3 and β 1→4 (McCandless and Craigie, 1979).

The structure of the repeating dissacharide in kappa-carrageenan is D-galactose 4-sulfate and 3,6-anhydro-D-galactose (Anderson et al., 1968a). Iota-carrageenan differs from kappa-carrageenan in that the 3,6-anhydro-D-galactose unit is 2-sulfated (Anderson et al., 1973). Lambda-carrageenan consists of D-galactose-2-sulfate and D-galactose-2,6-disulfate as the repeating units (Rees, 1963). The above structures are ideal and the naturally occurring molecules may vary as follows. Kappa- and iota-carrageenans may have occassional D-galactose-6-sulfate,

D-galactose 2,6-disulfate or non-sulfated residues (Dolan and Rees, 1965). It is possible to obtain carrageenan molecules which are close to the ideal structures as evidenced by infrared spectroscopy. Figure 2 illustrates these three types of carrageenan.

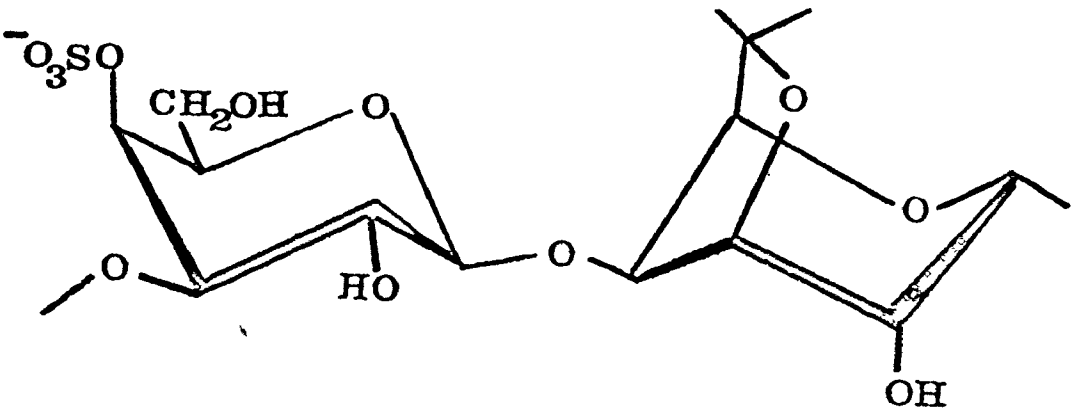
X-ray diffraction studies indicate that kappa- and iota-carrageenans form a double helix in solution (Mueller and Rees, 1968). This double helix structure explains the behaviour of carrageenans in aqueous solution. Both kappa- and iota-carrageenans form gels in the presence of K^+, Rb^+, Cs^+, or NH_4^+ but not of Li^+ or Na^+ (Rees, 1969). Because of this gelling capacity carrageenans are used for texture in many manufactured foods (Rees, 1970). Lambda-carrageenan is viscous in aqueous solution but does not gel (Rees, 1969).

Iota-carrageenans can be extracted in relatively pure form. Because kappa- and lambda-carrageenans are produced by two phases of plants of the same species (McCandless et al., 1973) either preparations must be fractionated or the plants must be separated as to phase prior to extraction. In the ensuing discussion unfractionated-carrageenan refers to a mixture of kappa- and lambda-carrageenans or unfractionated iota-carrageenan.

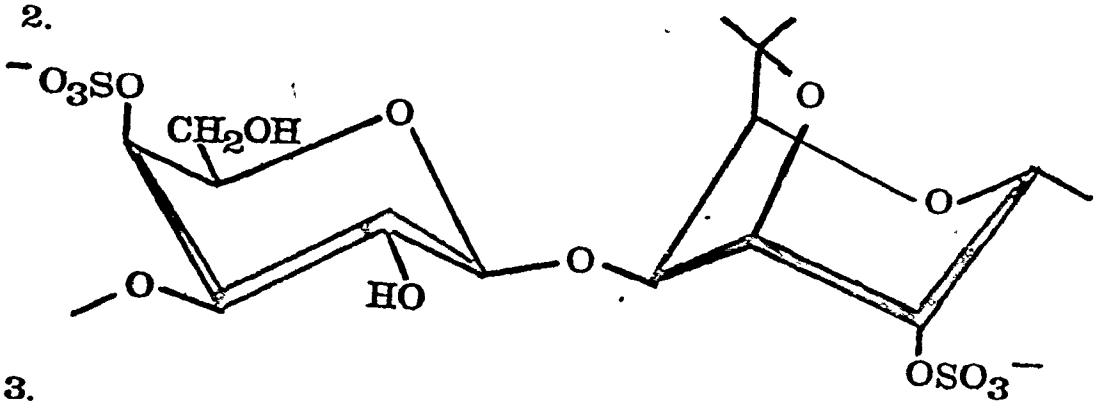
FIGURE 2. REPEATING DISACCHARIDE UNITS OF CARRAGEENANS

1. kappa-carrageenan
2. iota-carrageenan
3. lambda-carrageenan

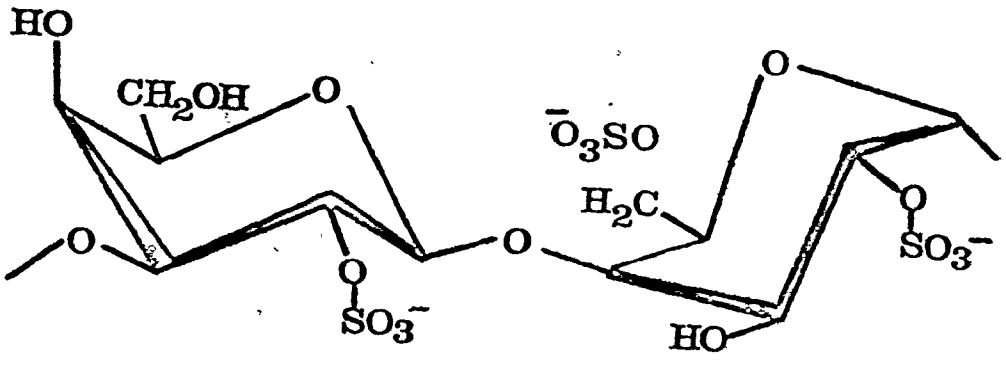
1.


$^{-}O_3SO$
CH_2OH
O
O
HO
O
O
O
OH

2.


$^{-}O_3SO$
CH_2OH
O
O
HO
O
O
O
OSO_3^{-}

3.


HO
CH_2OH
O
O
O
SO_3^{-}
$^{-}O_3SO$
H_2C
O
O
SO_3^{-}
HO

1.3.2 Effects on the Immune Response

Carrageenans have many effects on the immune response *in vivo* and *in vitro*. As the following discussion will show there is considerable controversy concerning the action of carrageenan on a variety of immune responses.

Table 4 gives a list of some immunosuppressive activities of carrageenans and table 5 lists some immunostimulatory properties of carrageenans. A comparison of tables 4 and 5 indicates that carrageenans can stimulate and suppress the same immune response.

Delayed hypersensitivity reactions were suppressed by high doses of carrageenan (Bice *et al.*, 1971; Schwartz and Leskowitz, 1969; Boros and Schwartz, 1975) and enhanced by low doses of carrageenan (Mizushima *et al.*, 1974a). The mechanism of suppression or enhancement was unclear in all of these studies. Bice *et al.* (1971) and Schwartz and Leskowitz (1969) suggested that carrageenan suppressed delayed hypersensitivity reactions by killing macrophages, however they did not present direct evidence to support their hypothesis. Because carrageenan can act as an antigen for delayed hypersensitivity reactions (Mizushima *et al.*, 1974b) and since carrageenan also

TABLE 4. IMMUNOSUPPRESSIVE ACTIVITIES OF CARRAGEENAN

Activity	Types of Carrageenan Used	References
1. suppression of delayed hypersensitivity	unfractionated, lambda, kappa	Bice *et al.*, 1971; Schwartz and Leskowitz, 1969; Boros and Schwartz, 1975
2. suppression of hybrid resistance, *in vivo*	unfractionated, lambda, kappa, iota	Yung and Cudkowicz, 1977; Lotzova *et al.*, 1975; Cudkowicz and Yung, 1977; Buurman and Van Bruggen, 1976
in vitro	lambda, kappa, iota	Yung and Cudkowicz, 1978a; Yung and Cudkowicz, 1978b
3. enhancement of tumor growth	unfractionated, iota	Rumjanek and Brent, 1978; Thomson and Fowler, 1977
4. suppression of allograft rejection	unfractionated	Rumjanek and Brent, 1978
5. suppression of antibody production	unfractionated lambda, kappa, iota	Aschheim and Raffel, 1972; Thomson *et al.*, 1976; Rumjanek *et al.*, 1977; Ishizaka *et al.*, 1977; Ishizaka *et al.*, 1978

CONTINUED...

TABLE 4 continued...

Activity	Types of Carrageenan Used	References
6. impairment of macrophage viability, *in vivo*	unfractionated	Lukic *et al.*, 1975
in vitro	unfractionated	Allison *et al.*, 1966; Catanzaro *et al.*, 1971
7. impairment of phagocytosis by macrophages	unfractionated	Fowler and Thomson, 1978
8. inhibition of the mitogenic response to phytohemagglutinin	lambda	Pawlec and Brons, 1978
9. suppression of the mixed-lymphocyte reaction	lambda	Pawlec and Brons, 1978
10. impairment of lymphocyte recirculation	unfractionated	Rumjanek and Brent, 1978

TABLE 5. IMMUNOSTIMULATORY ACTIVITIES OF CARRAGEENAN

Activity	Types of Carrageenan Used	References
1. enhancement of delayed hypersensitivity	kappa	Mizushima et al., 1974a
2. suppression of tumor growth	unfractionated	Shinoda et al., 1977
3. enhancement of antibody production	unfractionated, lambda	Turner and Higginbotham, 1977; Becker and Rudbach, 1979; Richou et al., 1968; Richou et al., 1970
4. augmentation of phagocytosis by macrophages	lambda	Aschheim and Raffel, 1972
5. stimulation of the mitogenic response to phytohemagglutinin	unfractionated, lambda	Pawlec and Brons, 1978; Blitstein-Willinger et al., 1976
6. stimulation of cytotoxic T-cell activity	lambda	Pawlec and Brons, 1978
7. mitogenic stimulation of B cells	iota	Quan et al., 1978
8. stimulation of lymphocyte recirculation	unfractionated	Fowler and Thomson, 1978

has inflammatory properties (DiRosa, 1972), it is difficult to interpret experiments which involve the modulating effects of carrageenan on delayed hypersensitivity.

There seems to be general agreement that carrageenan suppresses hybrid resistance (Yung and Cudkowicz, 1977; Lotzova et al., 1975; Cudkowicz and Yung, 1977; Buurman, 1976; Yung and Cudkowicz, 1978a; Yung and Cudkowicz, 1978b). Hybrid resistance is the tendency of irradiated F1 mice to resist parental bone marrow grafts. This phenomenon is believed to be due to the presence of hemopoietic histocompatibility antigens on the homozygous parental cells (Lotzova et al., 1975). The mechanism of this resistance may involve natural killer cells (Lotzova and Savary, 1977; see Heberman and Holden (1978) for a review of natural killer cells).

Carrageenan effectively suppressed hybrid resistance over a dose range of 0.5-2.0 mg i.v. (Cudkowicz, 1977) and 20 mg i.p. (Buurman and Van Bruggen, 1976). Although several studies suggested the mode of action was due to destruction of macrophages (Yung and Cudkowicz, 1977; Cudkowicz and Yung, 1977; Lotzova et al., 1975), Buurman (1976) found no significant visible destruction of these cells. In addition, polyvinyl-N-oxide which prevented the hybrid resistance inhibiting effects of silica (an

anti-macrophage agent) had no effect on carrageenan-mediated suppression (Yung and Cudkowicz, 1977; Lotzova et al., 1975; Cudkowicz and Yung, 1977). Carrageenan and silica did have additive suppressive effects (Cudkowicz and Yung, 1977) and therefore it was argued that carrageenan acted on macrophages by a different mechanism (Lotzova et al., 1975) or at a different stage of differentiation (Cudkowicz and Yung, 1977) than silica. Alternately, carrageenan-mediated suppression of hybrid resistance in vitro may be due to the induction of a suppressor cell (Yung and Cudkowicz, 1978a; Yung and Cudkowicz, 1978b). The carrageenan-induced suppressor cell was found to be a non-T cell which was adherent but not phagocytic (Yung and Cudkowicz, 1978b). Yung and Cudkowicz (1978b) suggested that the suppressor cells belonged to the monocyte-macrophage lineage.

A very high dose of carrageenan, 40 mg injected s.c. into a mouse, has been reported to inhibit the rejection of foreign skin grafts (Rumjanek and Brent, 1978). Rumjanek and Brent (1978) also found that this same dose of unfractionated-carrageenan, administered s.c., potentiated the growth of an allogeneic tumor. Enhancement of tumor growth by carrageenan treatment has also been demonstrated by Thomson and Fowler (1977). They found that when mice were pretreated with 4 mg

iota-carrageenan the growth of subsequently injected syngeneic tumor cells was enhanced. In contrast to the above findings 2 mg unfractionated-carrageenan, given with tumor antiserum, reduced the growth of an ascitic tumor but had no effect on a solid tumor (Shinoda, 1977). The above experiments seem to depend on timing for their effect. For instance, Thomson and Fowler (1977) found that when mice were treated with carrageenan after the injection of tumor cells there was no effect on tumor growth. This concept will be discussed in more detail (see below).

As shown in table 4, carrageenan has been reported to be cytotoxic for macrophages *in vivo* (Lukic *et al.*, 1975) and *in vitro* (Allison *et al.*, 1966; Catanzaro *et al.*, 1971). However, Simon and Jones (1979), in a recent paper, found that doses of carrageenan up to 1,600 $\mu g\ ml^{-1}$ *in vitro* were not cytotoxic for macrophages. Turner and Higginbotham (1977) reported that doses of carrageenan as high as 20 mg/mouse did not kill macrophages *in vivo*. Similarly, carrageenan has been reported to impair macrophage phagocytosis by some investigators (Fowler and Thomson, 1978), or to have no effect on phagocytosis by macrophages (Turner and Higginbotham, 1977; Simon and Jones, 1979), or to enhance macrophage phagocytosis

(Aschheim and Raffel, 1972). In view of the evidence, particularly that presented by Simon and Jones (1979), it appears that carrageenan is not selectively cytotoxic for macrophages *in vitro*. Since carrageenan is engulfed by macrophages and therefore may cause blockade of the mononuclear phagocyte system the effect of the polysaccharide on phagocytosis may depend on carrageenan dose.

Carrageenan can also affect T cells *in vitro*. Carrageenan altered the phytohemagglutinin response of lymphocytes as measured by H^3-thymidine uptake. Fifty µg carrageenan, added with phytohemagglutinin to murine thymocytes, was found to have a synergistic effect on the phytohemagglutinin response (Blitstein-Willinger *et al.*, 1976). The authors suggested that carrageenan changed the properties of the thymocyte membrane making it more responsive to phytohemagglutinin. Another study looked at the effect of various procedures on the phytohemagglutinin response of pig peripheral blood cells (Pawlec and Brons, 1978). The authors found that the removal of adherent cells or treatment with silica had no effect on the response. However 1,000 µg ml^{-1} lambda-carrageenan enhanced the phytohemagglutinin response while 1 µg ml^{-1} lambda-carrageenan suppressed the response. Pawlec and Brons (1978) presented this as

evidence that carrageenan could act directly on lymphocytes. In contrast Bice et al. (1971) found that carrageenan in the dose range of 50-500 μg ml^{-1} had no effect on the phytohemagglutinin response of murine thymocytes. It is unclear whether or not carrageenan can modulate the mitogenic response of T cells to phytohemagglutinin. This modulatory property of carrageenan seems to be dependent on the dose of carrageenan used and possibly the type of carrageenan and source of T cells.

Carrageenan can also affect B cells. Iota-carrageenan (200 μg ml^{-1}) augmented the uptake of H^3-thymidine by murine B cells or a mixture of B and T cells but not by T cells alone (Quan et al., 1978). This effect was observed with lymphocytes from several mouse strains and the removal of adherent cells had no effect on the phenomenon. Quan et al. (1978) concluded that carrageenan was a B-cell mitogen with similar properties to dextran sulfate.

Since carrageenans can affect macrophages, T cells, and B cells it is not surprising that these molecules can modulate humoral immune responses. Carrageenan has been found to have suppressive and stimulatory effects on humoral immunity (tables 4 and 5). Carrageenan suppressed the anti-SRBC response of mice (Aschheim and Raffel,

1972; Thomson et al., 1976; Rumjanek et al., 1977). All three types of carrageenan were found to be effective suppressors of this response with the order of potency being lambda- > iota- > kappa-carrageenan (Thomson et al., 1976). Carrageenan treatment prior to or simultaneous with SRBC was suppressive but administration of the polysaccharide 24 hr after antigen had no effect (Thomson et al., 1976). Thomson et al. (1976) showed that doses of carrageenan in the range of 0.25-5.0 mg were effective at inhibiting the response. In vitro studies showed that a suppressive adherent cell was induced in carrageenan-treated animals (Rumjanek et al., 1977).

Ishizaka et al. (1977,1978) looked at the effect of carrageenan on the humoral immune response in more detail. They injected mice with 0.5 mg unfractionated-carrageenan on days -7, -5, -3, and -1 and looked at the antibody response to T-cell dependent and T-cell independent antigens. They found that carrageenan treatment suppressed the response to the T-cell dependent antigen (SRBC) and either had little effect or enhanced the response to the T-cell independent antigen (trinitrophenylated-Ficoll) (Ishizaka et al., 1977). Subsequently (Ishizaka et al., 1978) they looked at the effect of this treatment regimen on different strains of mice. It was found that carrageenan treatment enhanced

the antibody response of C57BL/6 mice to dinitrophenylated-bovine gamma-globulin and it suppressed the antibody response of C3H mice to the same antigen. C57BL/6 mice are normally low responders to dinitrophenylated-bovine gamma-globulin and C3H mice are usually high responders.

There are also some reports of carrageenans enhancing the humoral immune response. Turner and Higginbotham (1977) reported that lambda-carrageenan (0.5 mg) given with SRBC augmented the anti-SRBC antibody titer. If the animals were pretreated with carrageenan there was no effect on the titer. Five mg unfractionated-carrageenan given 6 hr prior to SRBC enhanced the serum IgG and IgM titers to SRBC (Becker and Rudbach, 1979). This treatment had no effect on the anti-lipopolysaccharide response or on the secondary response to either antigen. Two studies by Richou et al. (1968, 1970) showed that unfractionated-carrageenan elevated the antitoxin titer to staphylococcal anatoxin in rabbits and mice. In these experiments the carrageenan was administered with the antigen.

In all of the experiments where carrageenan was found to suppress the humoral immune response, except one (Thomson et al., 1976), the carrageenan had to be administered prior to (24 hr) antigen in order to suppress the response. In contrast, augmentation of the humoral

immune response was observed when carrageenan was given with antigen. Other substances with adjuvant properties have also been reported to suppress the immune response when given prior to antigen (Leclerc et al., 1979; Mathe et al., 1975). Carrageenan pretreated mice had a nonadherent suppressive cell which inhibited the antibody response in vitro (Rumjanek et al., 1977). This cell seems similar to the one which suppressed hybrid resistance in vitro (Yung and Cudkowicz, 1978b). It may be that, in the absence of antigen, carrageenan can induce a non-specific macrophage-like suppressor cell. Ishizaka et al.'s (1978) results with high and low responder mice suggest that carrageenan acts on some regulatory cell involved in humoral immunity possibly a T cell (Eardley et al., 1976).

Carrageenans have a variety of other activities in biological systems which may be relevant to their effects on the immune response. These properties have been reviewed by DiRosa (1972) and include the following: granuloma formation, edema formation, kinin release in vitro and possibly in vivo, inhibition of the complement system in vitro and in vivo, inhibition of peptic ulcer formation, colonic ulceration, and humoral immunogenicity when appropriately presented.

In summary, it is obvious from the above

discussion that carrageenans can modulate the immune response *in vivo* and *in vitro*. It is equally apparent that there is little agreement regarding the direction of this modulation (stimulatory or suppressive) and the potential mode of action of the observed effect. It seems that the type and dose of carrageenan are important factors for determining the subsequent effect(s) of the polysaccharide. The time of carrageenan administration, relative to antigen, and the genetic background of the experimental animals may also be important.

1.4 Scope

In broad terms this thesis concerns the control of the immune response. More specifically, carrageenans were used to enhance the humoral immune response and the mechanism of this enhancement was studied. This information suggests the use of carrageenans as immunologic adjuvants and should caution those who use the polysaccharide solely as an anti-macrophage agent. The data acquired from this thesis bear on our understanding of the regulation of the immune response.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Mice

C57BL/6 male mice, C57BL/6 female mice, and BALB/c female mice were purchased from Jackson Laboratories, Bar Harbour, Maine. BALB/c male mice were purchased from either Jackson Laboratories or Biobreeding, Ottawa, Ontario. BALB/c X C57BL/6 F1 mice were produced in the McMaster University animal facilities. The PFC assays were carried out with 6-8 week old mice. The mice, caged in groups of 3-6, were fed laboratory chow and allowed tap water *ad libitum*.

2.1.2 Carrageenans

The carrageenans used in these experiments were extracted and fractionated as described by McCandless *et al*. (1973). The carrageenan preparations listed in table 6 were characterized by infrared spectroscopy and

TABLE 6. TYPES AND SOURCES OF CARRAGEENAN

Type of Carrageenan	Laboratory Name	Plant Source	Laboratory Source
kappa	CC_{13}	Chondrus crispus	Dr. McCandless, McMaster Univ.
kappa	T_4	C. crispus	Dr. McCandless, McMaster Univ.
lambda	T_3	Iridaea cordata	Dr. McCandless, McMaster Univ.
iota	6060	Eucheuma spinosum	Marine Colloids, Rockland, Maine

chemical analysis (these methods are described by McCandless et al. (1973)). Stock solutions of carrageenan were made by dissolving 100 mg carrageenan in 10 ml distilled water, and sterilized by autoclaving. This sterile solution was dispensed in volumes of one or two ml into sterile containers and stored at -60°C for future use. Dilutions of this stock solution were made with sterile distilled water.

2.1.3 Antigens

Horse erythrocytes (HRBC) and SRBC, in Alsever solution, were purchased from Woodlynn Laboratories, Guelph, Ontario. Both antigens were thoroughly washed with Hanks' saline solution prior to use. After centrifugation, the packed red blood cells were resuspended in Hanks' saline solution to the desired concentration.

2.2 Methods

2.2.1 Plaque-Forming Cell Assay

This technique was first described by Jerne and Nordin in 1963 and has been modified extensively

since then (reviewed by Jerne et al. (1974)). The PFC assay measures the number of cells which produce antibody of a particular specificity. This technique can be used to measure the class, subclass, and avidity of the antibody released by the PFC.

In its most basic form the method is used to detect cells producing antibody of the IgM class which is specific for erythrocyte antigens. To perform this assay, an animal is immunized with foreign red blood cells (e.g. SRBC in a mouse) and 3-7 days later its spleen is removed and a suspension is made of the splenocytes. These cells are added to a mixture of melted agar and SRBC, poured onto a petri dish or glass slide, and incubated at 37°C for 1 hr. During the 1 hr incubation, antibodies specific for SRBC are released and bind to the SRBC present nearby in the agar mixture. After 1 hr, complement is added to the plates which are then incubated for an additional 30 min. During this second incubation, antibody which has bound to SRBC activates complement causing lysis of SRBC. The hemoglobin may be washed away leaving a clear area or plaque. Each plaque is due to the presence of one PFC. Thus, the number of PFC in a given organ or tissue culture can be determined. Under the conditions described above only antibody of the IgM class causes efficient lysis of

SRBC. These are called direct PFC. The addition of appropriate antisera allows detection of PFC which produce antibody of other classes (indirect PFC). The PFC assay is versatile since numberous antigens can be chemically coupled to the SRBC and thus the antibody response to a variety of T-cell dependent and T-cell independent antigens can be measured.

2.2.1.1 Procedure

Mice were immunized with SRBC or HRBC ($5x10^7$ cells per mouse unless otherwise indicated) i.p. in a volume of 0.1 ml. Several days later (five unless otherwise indicated) the mice were killed by cervical dislocation and their spleens were removed. The spleens were placed in sterile Hanks' saline solution (see table 7). A spleen cell suspension was made by teasing the spleens apart with fine forceps and scissors and repeated aspiration with a Pasteur pipette before transferring into a test tube. A portion of the cell suspension was diluted in trypan blue (BDH Chemicals, Poole, England) (2% stock in Hanks' saline solution) to determine the number of viable (dye excluding) cells. A minimum of 100 viable nucleated cells were counted using a Nebauer improved hemocytometer. The cells then were diluted in

TABLE 7. HANKS' SALINE SOLUTION (a)

Chemical	Concentration (gl^{-1})
sodium chloride	8.00
calcium chloride	0.20
magnesium sulfate	0.20
potassium chloride	0.40
potassium di-hydrogen phosphate	0.10
sodium bicarbonate	1.27
glucose	2.00

(a) The saline was sterilized by filtration. This recipe was obtained from Hudson and Hay (1976).

Hanks' saline solution to a concentration of $5x10^5$ viable cells ml^{-1}. All manipulations done with the spleens were in ice cold Hanks' saline solution and the splenocytes were stored in Hanks' saline solution at 4^oC.

In order to determine the number of PFC, an aliquot (0.1 ml, $5x10^4$ cells) of spleen cell suspension was added to duplicate agar coated petri dishes which had been prepared by adding one ml of melted agar (Difco Bacto-Agar, Difco Laboratories, Detroit, Michigan) (1.4% $^W/_V$ in Hanks' saline solution) to each 60x15 mm plastic petri dish (Falcon, Oxnard, California). The agar was evenly distributed by tilting the plate and allowed to solidify with the plate on a level surface. After the spleen cells had been added to the plate a mixture of SRBC and melted agar was poured onto the plate and evenly distributed. The mixture was prepared by adding 25 µl of a 1:3 ($^V/_V$) suspension of SRBC to 0.5 ml melted agar (0.7%, $^W/_V$) containing 0.5 mg ml^{-1} DEAE dextran (approximate molecular weight 500,000, Pharmacia Fine Chemicals, Uppsala, Sweden). The agar had been previously dispensed into small test tubes in a 50^oC water bath. After the SRBC and melted agar were added to the plates they were incubated in a humidified CO_2 incubator (37^oC, 5% CO_2 in air) for one hr. The plates were then removed from the incubator and one ml diluted

guinea pig complement (Gibco, Grand Island, N.Y.) (1:10, $^{v}/_{v}$ in Hanks' saline solution) was carefully poured onto each plate. This dilution of complement was found to be optimal for developing PFC (see figure 3). After a second incubation (30 min.) the excess complement was removed and the plaques were counted. Figure 4 is a diagram of the basic assay procedure. Only direct PFC were determined in most experiments. Indirect PFC were detected by adding one ml of rabbit anti-mouse IgG (diluted 1:200 in Hanks' saline solution) to the plates after the first incubation. The plates were then removed from the incubator, excess antisera was washed off, and complement was added as described above. The rabbit anti-mouse Ig (Miles Laboratories, Elkhart, Indiana) was extensively absorbed with SRBC and titrated to determine the optimal dilution for developing indirect PFC.

2.2.2 Design of *in vivo* Experiments

Most experiments consisted of four treatment groups; which received:

1. Hanks' saline solution and distilled water
2. carrageenan and Hanks' saline solution
3. SRBC and distilled water

FIGURE 3. The effect of different concentrations (%, $^{v}/_{v}$) of complement on the number of observed PFC. BALB/c mice were immunized with SRBC and the PFC assay was performed five days later. The results are expressed as the geometric mean of two plates per point.

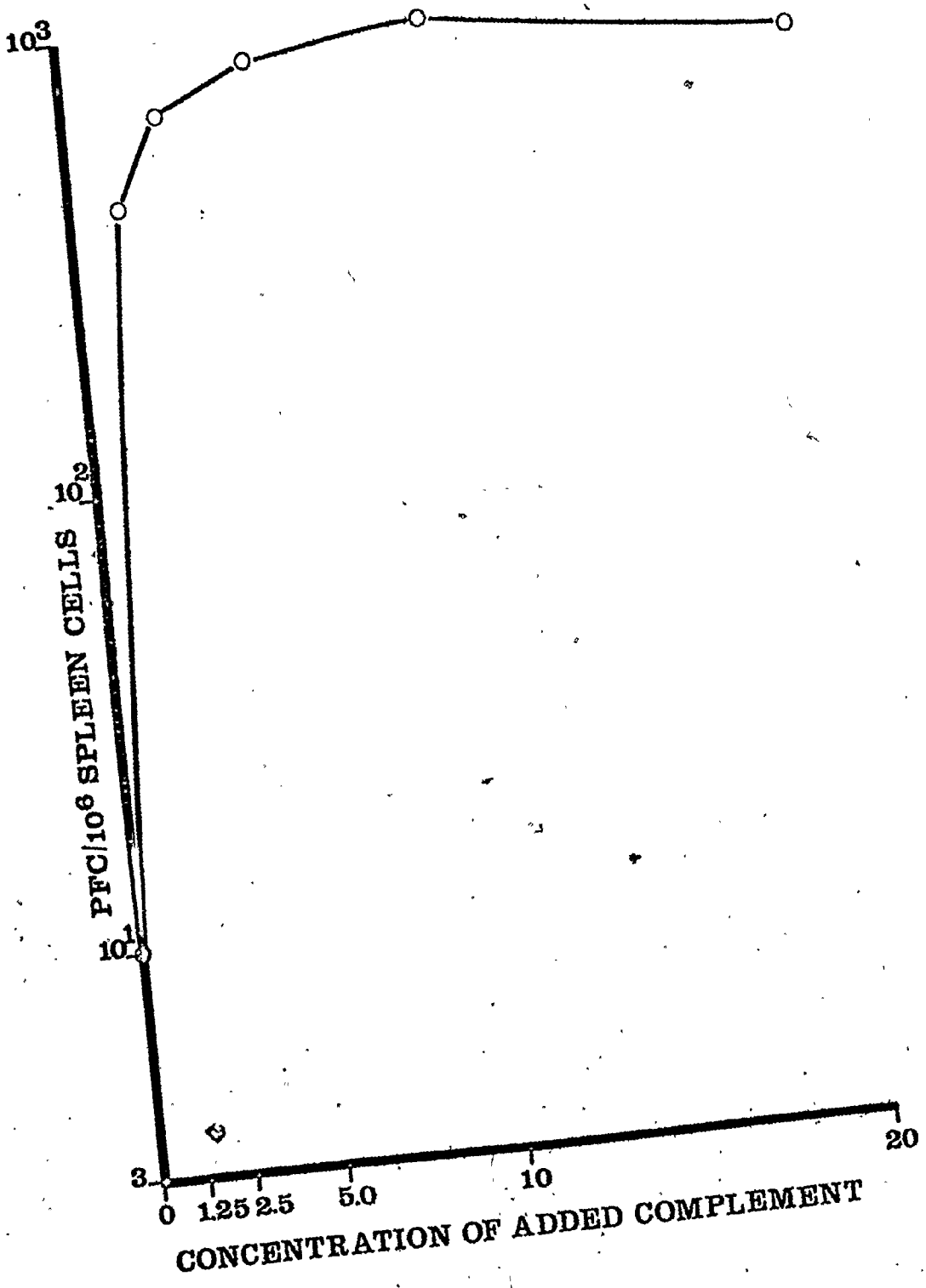
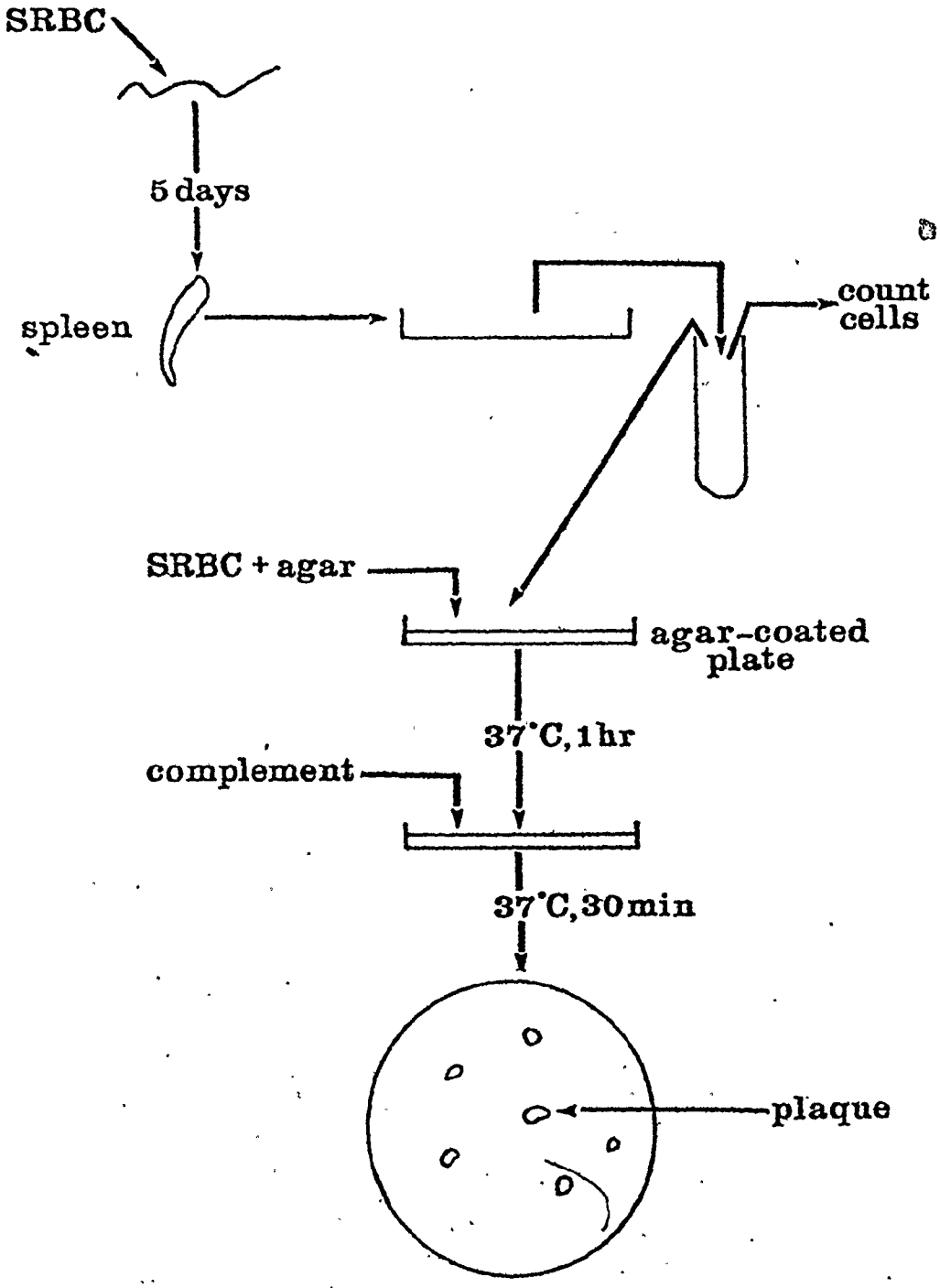

10^3
10^2
10^1
3
PFC/10^6 SPLEEN CELLS
0
1.25
2.5
5.0
10
20
CONCENTRATION OF ADDED COMPLEMENT

Figure 4 A Schematic Diagram of the PFC Assay



4. carrageenan and SRBC

All injections were given in a volume of 0.1 ml and were administered i.p. Each injection was given using a separate syringe. Each treatment group consisted of 3-6 randomly selected mice. The spleens from each treatment group were pooled in some experiments and assayed individually in others.

2.2.3 Tube Culture Experiments

Spleen cells from normal mice ($2x10^6$/culture) were incubated with SRBC ($1x10^6$/culture) in 17x100 mm polypropylene tubes (Falcon, Oxnard, California, cat. no. 2059). The cells were cultured in minimal essential alpha medium (Grand Island Biological Co., Grand Island, N.Y., cat. no. 320-2571) supplemented with 100 units penicillin and 100 µg streptomycin per ml (penicillin/streptomycin solution, GIBCO, Grand Island, N.Y.), and $5x10^{-5}$M 2-mercaptoethanol (Sigma, St. Louis, Mo.). This recipe will be referred to as basic medium. In the tube cultures 10% ($^v/_v$) fetal calf serum (Gibco, lot no. A585626) was added to the basic medium.

The tube cultures were divided into the following treatment groups:

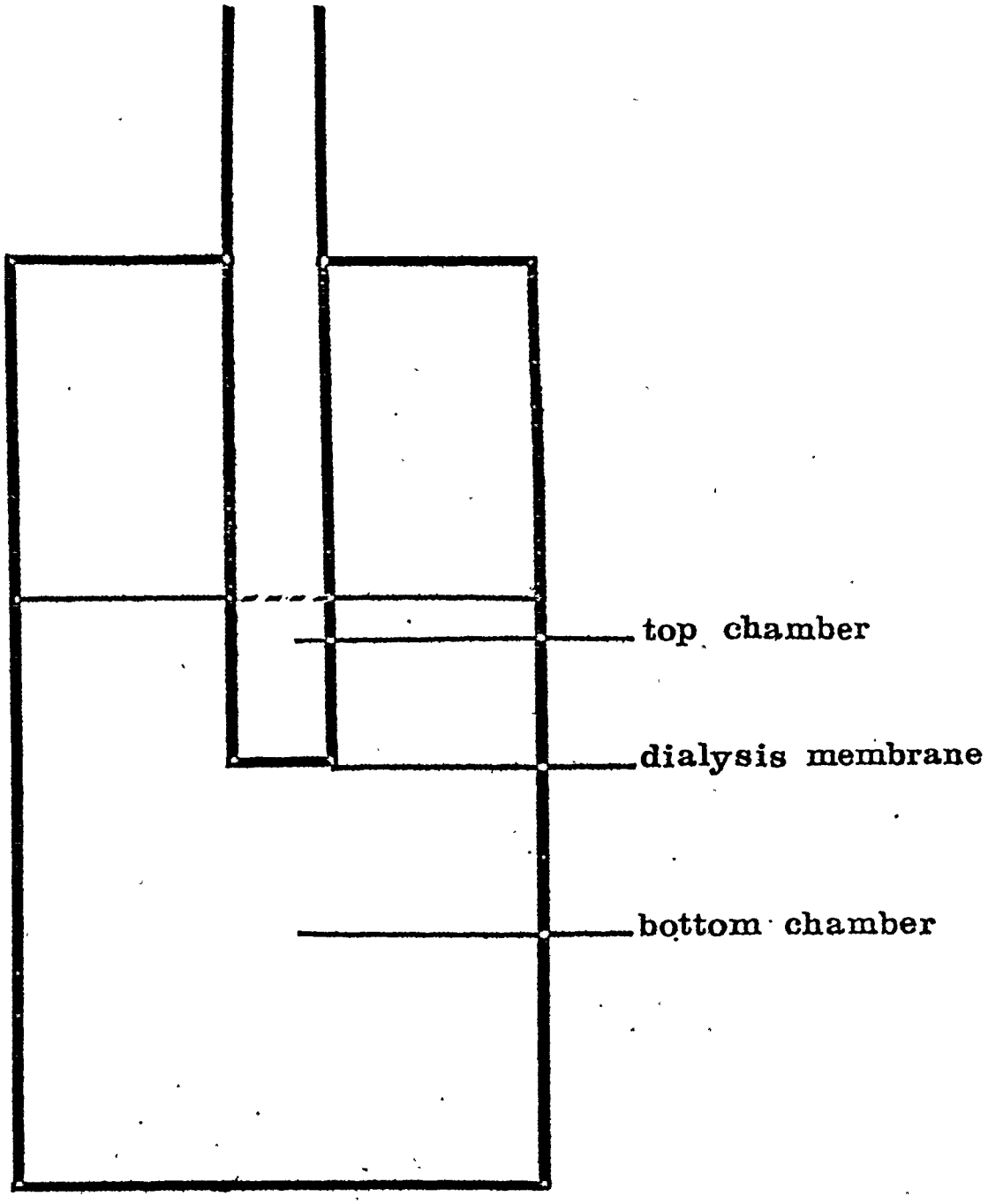
1. spleen cells + 0.1 ml Hanks' saline solution + 0.1 ml distilled water
2. spleen cells + 0.1 ml SRBC + 0.1 ml distilled water
3. spleen cells + 0.1 ml Hanks' saline solution + 0.1 ml carrageenan
4. spleen cells + 0.1 ml SRBC + 0.1 ml carrageenan

Each culture was performed in a final volume of 2 ml and was incubated for five days at 37°C in a humidified CO_2 incubator. The number of PFC per culture was determined using the Jerne plaque assay, as described in section 2.2.1.1 with the exception that all manipulations with cells were done in basic medium instead of Hanks' saline solution.

2.2.4 Marbrook Vessel Experiments

Marbrook vessels were purchased from Belco Glass, Vineland, N.J. and a number were kindly provided by Dr. R. A. Phillips, Ontario Cancer Institute, Toronto. The basic design of these glass vessels is shown in figure 5. The Marbrook vessel consists of two chambers for culturing cells, a small top chamber separated from a larger bottom chamber by a dialysis membrane.

Figure 5 Diagram of a Marbrook Vessel



2.2.4.1 Procedure

The dialysis membrane to separate the top and bottom chambers was prepared by soaking for 1 hr in distilled water. A small piece of treated membrane was then stretched over the open bottom end of the top chamber and secured in place by a silastic o-ring which had also been soaked and boiled with the dialysis membrane. The top of the Marbrook vessel was then covered with foil and the completed unit was sterilized by autoclaving. Various numbers of untreated spleen cells were cultured in the top chamber together with SRBC in a total volume of 1 ml. The cells were cultured in basic medium supplemented with 10% fetal calf serum (Gibco, lot no. A585626). The bottom chamber contained 12 ml basic medium without fetal calf serum. The Marbrook vessels were incubated for five days at 37°C in a humidified CO_2 incubator. The number of PFC developing in the top chamber was then assayed using the Jerne plaque method. Cultures which had "leaky" dialysis membranes were not assayed. Marbrook vessels were thoroughly washed in chromic acid prior to being reused.

2.2.4.2 Fractionation of Cells

2.2.4.2a Depletion of T cells

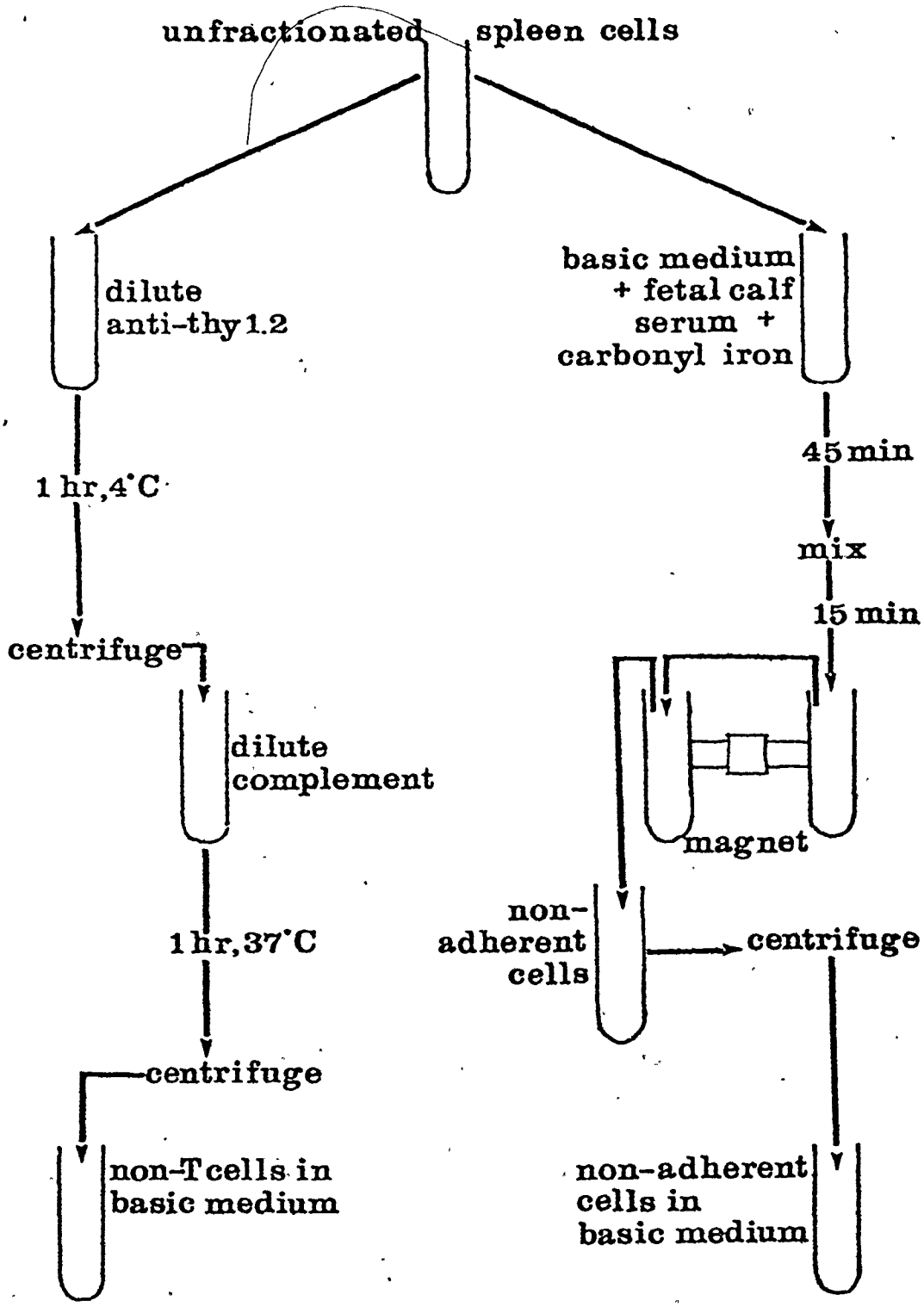
The medium used in this procedure was basic medium supplemented with 0.2% BSA (Cohn's fraction V, in phosphate buffered saline solution). Spleen cells ($100x10^6$) in 2.5 ml medium were added to 2.5 diluted anti-thy 1.2 (AKR anti-CBA thymus, a gift from Dr. M. McDermott). The diluted anti-thy 1.2 was prepared by adding 25 µl antiserum to 2.475 ml BSA-supplemented basic medium. This mixture was sterilized by Millipore filtration. Once the cells had been added to the dilute anti-thy 1.2 the mixture was incubated at 4^oC for 1 hr. After 1 hr the suspension was centrifuged at 1,000 g for 8 min. The supernatant fluid was discarded and the cell pellet was resuspended in 13 ml dilute rabbit complement. The complement (Low-tox-M rabbit complement, Cedarlane Laboratories, Hornby, Ont.) was purchased in 1 ml lyophilized volumes. Complement (1 ml) was added to 12 ml medium and the resulting solution was sterilized by Millipore filtration. The suspension of cells in dilute complement was incubated at 37^oC in a humidified CO_2 incubator for 1 hr. After this incubation the cells were centrifuged, resuspended in 2.5 ml basic

medium, and stored at $4^{o}C$. Forty to 60% of the viable cells were usually recovered and these are referred to as non-T cells.

2.2.4.2b Depletion of Adherent Cells

Spleen cells ($100x10^{6}$) in 5 mls basic medium supplemented with 10% fetal calf serum were added to one ml normal saline solution containing 0.5-1 gm carbonyl iron (particle size 3 μ, A. D. Mackay, N.Y.). The carbonyl iron-saline mixture was autoclaved before use. The spleen cell-carbonyl iron mixture was incubated at $37^{o}C$ in a humidified CO_2 incubator for 45 min and then gently mixed before being returned to the incubator for a further 15 min. A magnet was then held to the side of the test tube and the non-adherent cells were removed using a Pasteur pipette. A magnet was held to the side of the test tube containing the aspirated cells and the non-adherent cells were removed as before. The non-adherent cells were subsequently centrifuged and resuspended in 2.5 ml basic medium. The percentage recovery of viable cells was usually 10-50%. The recovered cells are referred to as non-adherent cells. Figure 6 gives a diagramatic scheme of the fractionation procedures used.

FIGURE 6. A diagramatic outline of the cell fractionation procedure used to obtain non-T and non-adherent cells.


unfractionated spleen cells
dilute anti-thy 1.2
1 hr, 4°C
centrifuge
dilute complement
1 hr, 37°C
centrifuge
non-T cells in basic medium
basic medium + fetal calf serum + carbonyl iron
45 min
mix
15 min
magnet
non-adherent cells
centrifuge
non-adherent cells in basic medium

2.2.4.3 Basic Design of Experiments

The basic plan of the Marbrook experiments was to put untreated cells together with SRBC in the top chamber and to assess the effect of cells, which had received different *in vivo* treatments, placed in bottom chamber on the PFC response of the top chamber cells. The bottom chamber contained: medium alone, untreated spleen cells, or spleen cells from mice which had been injected with carrageenan 6-12 hours prior to setting up the Marbrook vessel.

2.2.5 Statistics

All statistical calculations (except cell number data) were done with log transformed data. This transformation causes the PFC data to approximate a normal distribution (Gottlieb, 1974). Tests of statistical significance were done using the Student's two-tailed t-test unless otherwise indicated. The coefficient of correlation (r) was calculated according to the following formula:

$$r = \frac{\text{slope} \times \text{standard deviation of X}}{\text{standard deviation of Y}}$$

The statistical significance of r was assessed using a critical value for correlation coefficients table (Sokal and Rohlf, 1973).

Spearman's coefficient of rank correlation (r_s) was calculated as follows:

$$1 - \frac{6 \sum_i di^2}{(n-1)n(n+1)}$$

where di = the differences in ranks.

The statistical significance of r_s was tested using the following formula:

$$t = rs \quad \frac{n-2}{1-r_s^2}$$

The formulas for determining r_s were found in Steel and Torrie (1960).

Chapter 3

RESULTS

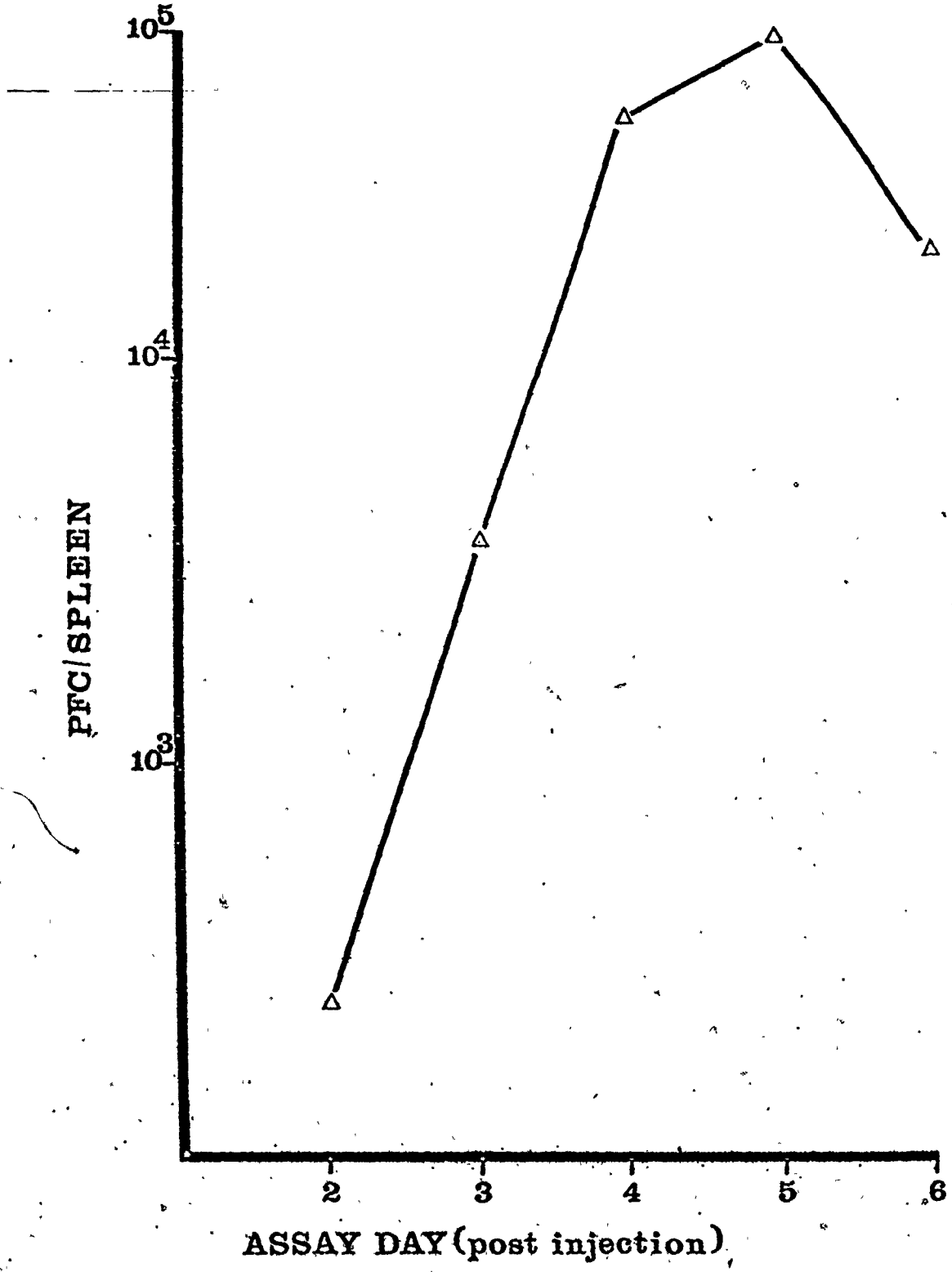
3.1 *In Vivo* Studies

The objectives of these studies were to determine if carrageenan had an adjuvant effect for the immune response *in vivo* and to characterize the conditions necessary for that effect.

3.1.1 Kinetics of the Normal Response

To determine when the PFC response was maximal, mice were injected with SRBC and the number of PFC developing at different times after immunization was determined. Figure 7 shows the results from one of these experiments using C57BL/6 mice. The response pattern was similar in BALB/c mice. Since the maximum numbers of PFC were found on day five in both strains, all subsequent assays were performed five days after immunization.

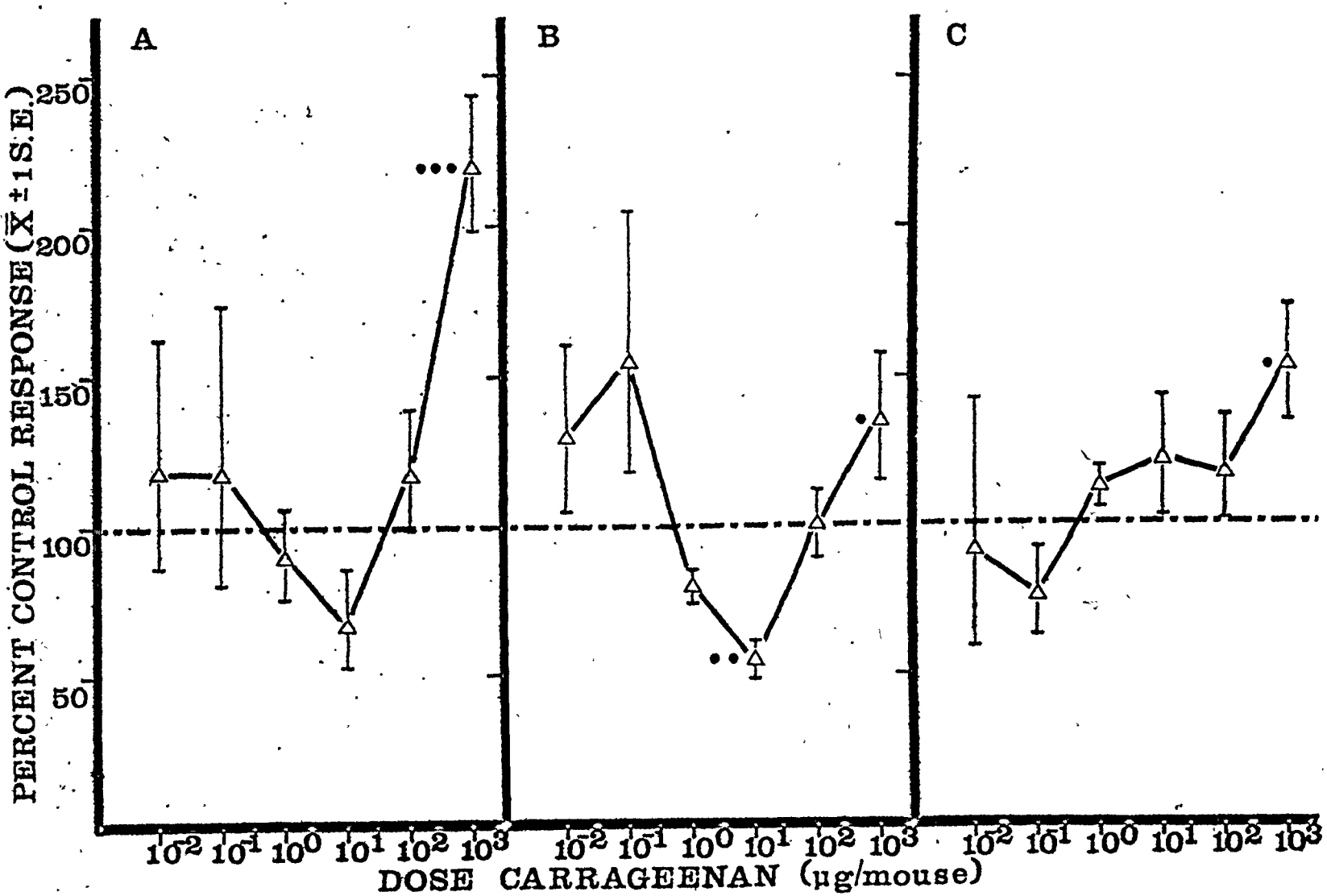
FIGURE 7. The number of PFC developing in C57BL/6 mice at different times after immunization. Five groups (five mice/group) of mice were immunized on day 0 with $5x10^7$ SRBC. One group of mice was killed on each day and the Jerne plaque assay was performed on the pooled spleens. The number of PFC/spleen is plotted on a $\log_{10}$ scale.


10^5
10^4
10^3
PFC/SPLEEN
2
3
4
5
6
ASSAY DAY (post injection)

3.1.2 Response of BALB/c Mice

Other groups of mice were then injected with SRBC and carrageenan and the number of PFC developing five days later was determined. Figure 8 shows results from a series of these experiments involving BALB/c mice. One mg kappa-carrageenan augmented the anti-SRBC response of BALB/c mice as measured by PFC/spleen (figure 8A) and by PFC/10^6 spleen cells (figure 8B). This dose of carrageenan also caused a significant increase in the number of spleen cells (figure 8C). An intermediate dose of kappa-carrageenan (10 µg) inhibited the antibody response to SRBC in terms of PFC/10^6 spleen cells. Although PFC/spleen were also reduced, the value was not statistically significant in this case since 10 µg kappa-carrageenan also increased the number of spleen cells thus raising the number of PFC/spleen. Low doses of carrageenan caused a modest increase in the number of PFC/10^6 spleen cells. It was found that higher doses of carrageenan (2 mg, 4 mg) were not as effective in stimulating the anti-SRBC response and the latter dose was found to be immunosuppressive. Because the aim of these experiments was to analyse the immunostimulatory effects of carrageenan on humoral immune responses, one mg kappa-carrageenan, which produced maximal enhancement,

FIGURE 8. The effect of different doses of kappa-carrageenan on the primary humoral immune response of BALB/c mice. A. PFC/spleen. B. PFC/10^6 spleen cells. C. cells/spleen. Groups of 3-6 mice were injected with the indicated dose of carrageenan together with $5x10^7$ SRBC. The control response is the response of animals injected with $5x10^7$ SRBC only. The results from several different experiments were pooled. Significant differences from controls are indicated: •••P = .0005, ••P = .005, •P = .025.


A
B
C
PERCENT CONTROL RESPONSE ($\bar{X}$ ± 1 S.E.)
250
200
150
100
50
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
DOSE CARRAGEENAN (µg/mouse)

was selected for further study.

Table 8 shows the effect of treatment with 1 mg kappa-carrageenan in more detail. It is clear that while carrageenan enhanced the response of BALB/c mice to SRBC it did not augment the background PFC response. These data suggest that the mechanism of this enhancement is not due to the polyclonal activation of antibody forming cells.

One possible explanation for this enhancement of the direct PFC response is that the indirect (IgG) response is inhibited. Carrageenan treatment could be preventing the switch from IgM to IgG production thus causing an increase in IgM production and a decrease in IgG production. The data shown in table 9 suggested that this was not the case. In both experiments kappa-carrageenan treatment increased the numbers of direct and indirect anti-SRBC PFC.

3.1.3 Strain Dependence of Carrageenan's Immunomodulatory Effects

The possibility that the effect of carrageenan treatment on the anti-SRBC response was dependent on the genetic background of the mice used was tested in the following experiments. Figure 9 shows the effect of injecting different doses of kappa-carrageenan on the

TABLE 8. THE EFFECT OF CARRAGEENAN TREATMENT ON THE PRIMARY HUMORAL IMMUNE RESPONSE OF BALB/c MICE

Treatment (a)	PFC/10^6 Spleen Cells (d)	PFC/Spleen (d)
none	2	54
carrageenan (b)	2	70
SRBC (c)	601(466-777)	27,261(19,863-37,415)
SRBC + carrageenan	1,352(1,161-1,575)	77,998(68,706-88,547)

(a) 5 mice/treatment group

(b) 1 mg kappa-carrageenan/mouse

(c) $5x10^7$ SRBC/mouse

(d) geometric mean ± 1 S.E.

TABLE 9. KAPPA-CARRAGEENAN (1 mg) INCREASES THE NUMBERS OF DIRECT AND INDIRECT PFC IN BALB/c MICE

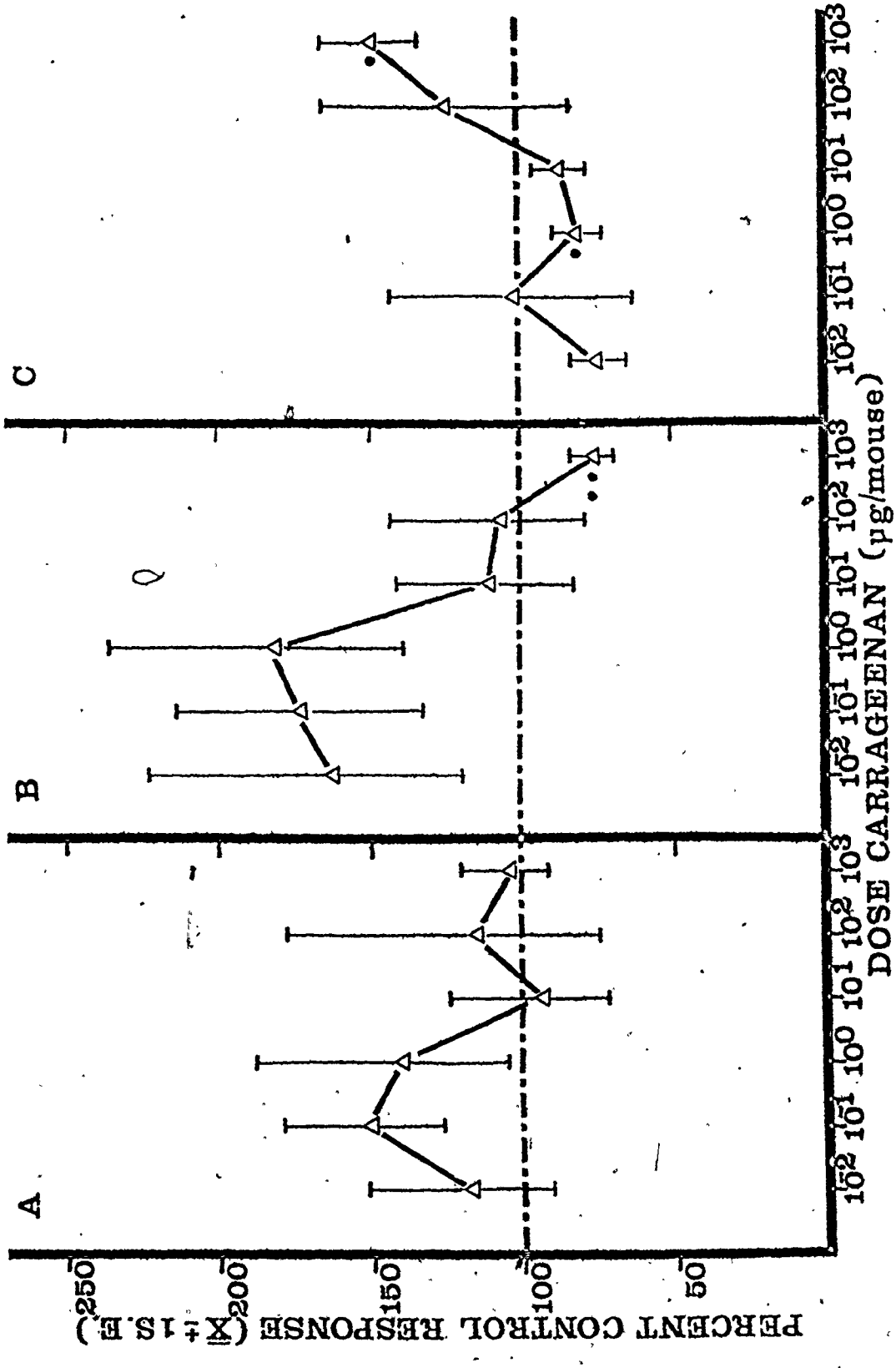
Treatment	PFC/Spleen					
	Expt. 1			Expt. 2		
	Direct	Indirect(a)	Total(b)	Direct	Indirect	Total
SRBC ($5x10^7$/ mouse)	57,040	29,492	86,532	32,782	31,510	64,292
Kappa-carrageenan + SRBC	227,394	138,783	366,177	67,636	74,000	141,636

(a) Total PFC - direct PFC

(b) Rabbit anti-mouse Ig (1:200) added to the plate

Groups of 5 mice were treated as indicated. The results from two separate experiments are shown.

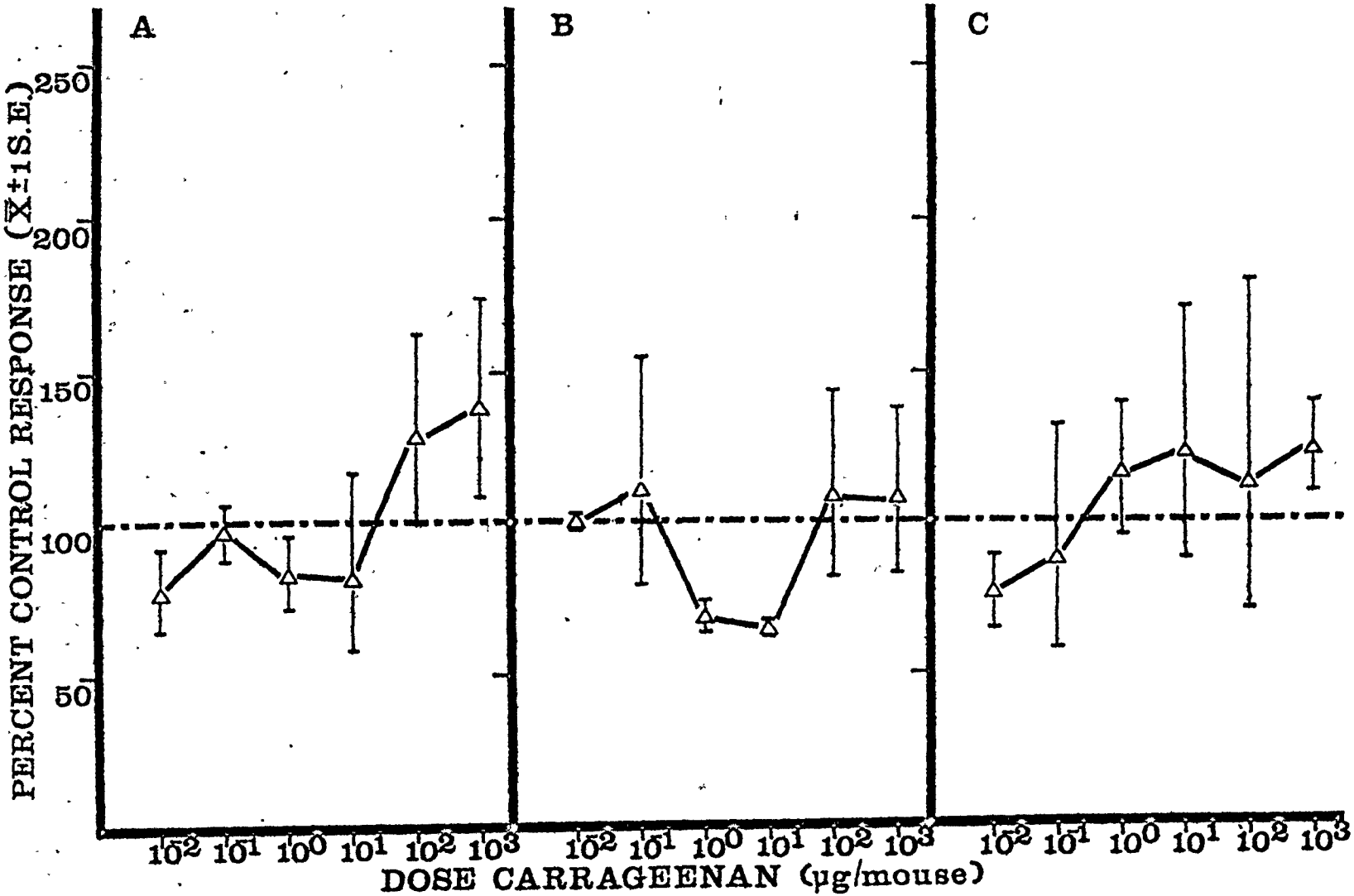
FIGURE 9. The effect of different doses of kappa-carrageenan on the primary humoral immune response of C57BL/6 mice. A. PFC/spleen. B. PFC/10^6 spleen cells. C. cells/spleen. Groups of 3-6 mice were injected with the indicated dose of carrageenan together with $5x10^7$ SRBC. The control response is the response of animals injected with $5x10^7$ SRBC only. Significant differences from controls are indicated: ••P = .005, •P = .025.


PERCENT CONTROL RESPONSE ($\bar{X} \pm 1$ S.E.)
250
200
150
100
50
A
B
C
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
DOSE CARRAGEENAN (µg/mouse)

anti-SRBC response of C57BL/6 mice. Except for the effect on cells/spleen, the dose response profile of C57BL/6 mice was dissimilar from that of BALB/c mice. Like BALB/c mice, low doses of kappa-carrageenan caused an increase in the anti-SRBC response. This enhancement of the immune response by low doses of kappa-carrageenan (1.0 and 0.1 µg) occurred in five of six experiments however it was not statistically significant ($P = 0.10$). Higher doses of kappa-carrageenan (1,000 µg), in contrast to the effect in BALB/c mice, proved suppressive in C57BL/6 mice in terms of PFC/10^6 spleen cells but because this dose of carrageenan caused a significant increase in the number of cells/spleen the net effect on the number of PFC/spleen was negligible. Higher doses of kappa-carrageenan (2 mg, 4 mg) suppressed the response in this strain. Since C57BL/6 mice responded to high doses of kappa-carrageenan differently from BALB/c mice it seemed that the effect of high doses of carrageenan was dependent on the genetic background of the mice. In an effort to test this idea the anti-SRBC response of BALB/c x C57BL/6 F_1 hybrid mice, injected with various doses of kappa-carrageenan, was examined.

Figure 10 shows the dose-response curve of the F_1 hybrid mice to kappa-carrageenan. In this strain 10 µg carrageenan is immunosuppressive (as seen in BALB/c mice).

FIGURE 10. The effect of different doses of kappa-carrageenan on the primary humoral immune response of BALB/c x C57BL/6 F_1 hybrid mice. A. PFC/spleen. B. PFC/10^6 spleen cells. C. cells/spleen. Groups of 3-6 mice were injected with the indicated dose of carrageenan together with $5x10^7$ SRBC. The control response is the response of animals injected with $5x10^7$ SRBC only. The results from several different experiments were pooled. Significant differences from controls are indicated: ••P = .005, •P = .025.


A
B
C
PERCENT CONTROL RESPONSE ($\bar{X}$±1S.E.)
250
200
150
100
50
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
DOSE CARRAGEENAN (µg/mouse)

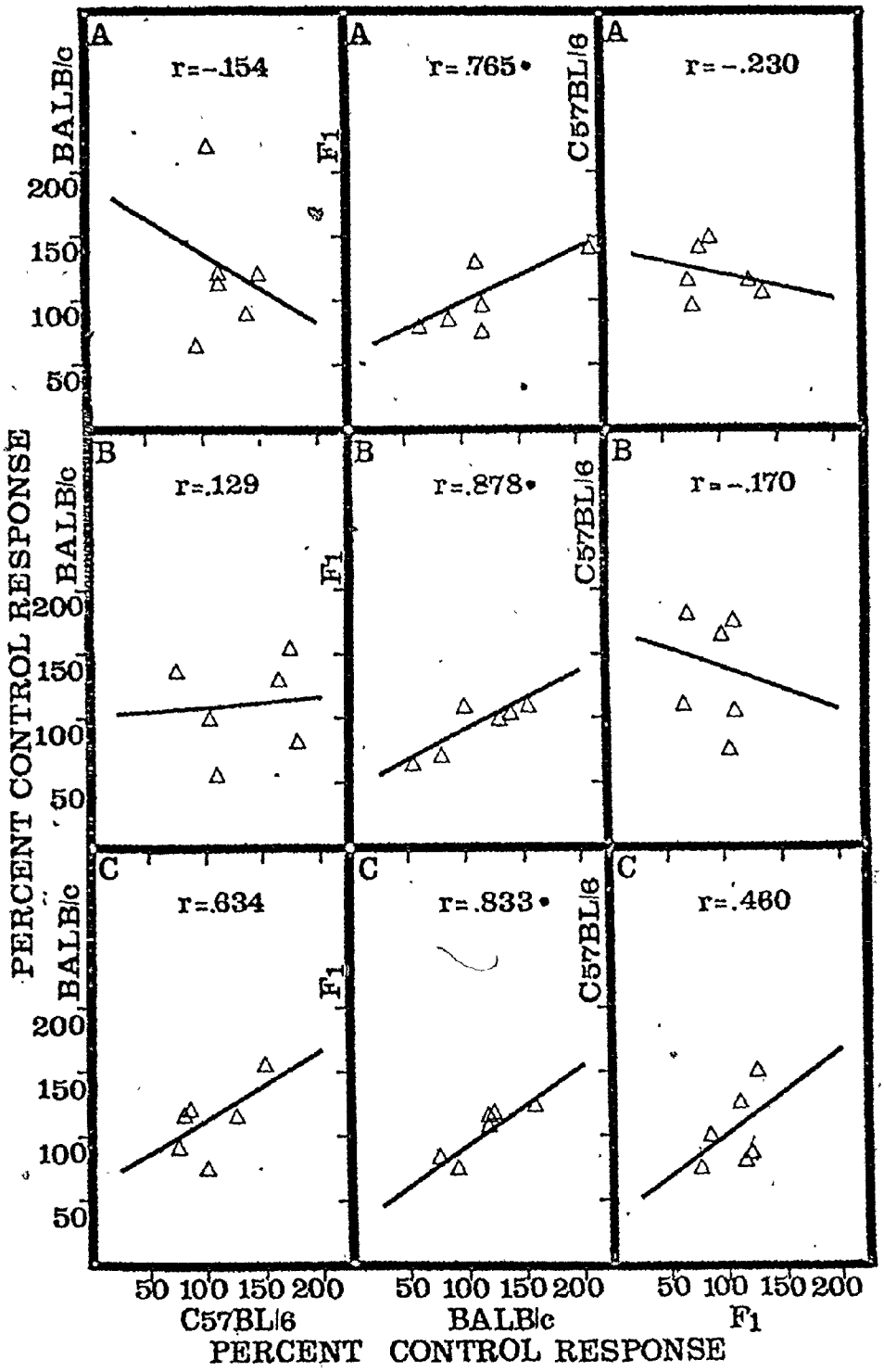
In an effort to relate the dose-response curves of F_1 hybrid mice to those of BALB/c and C57BL/6 mice the shapes of the dose-response curves among the three types of mice were compared. Figure 11 shows that the shape of the dose-response curves of F_1 hybrid mice are similar to the shape of BALB/c dose-response curves. In contrast, the dose-response curves of C57BL/6 mice differ from those given by F_1 hybrid mice, and from those of BALB/c mice. All three strains show a similar dose-response profile in terms of the effect of carrageenan treatment on the number of cells/spleen. These correlation curves suggest that F_1 hybrid mice respond to kappa-carrageenan in a manner which is similar to that of BALB/c mice at least at intermediate and high doses of carrageenan. Collectively these data suggest the adjuvant effect of kappa-carrageenan may have a genetic basis.

It was decided to further study the effect of carrageenan treatment on BALB/c mice since the enhancement of the immune response by 1.0 mg kappa-carrageenan in this strain was highly reproducible.

3.1.4 Importance of Timing on the Enhancing Effect of Carrageenan

Other investigators have shown that 1 mg carrageenan

FIGURE 11. The relationship between the dose response curves of BALB/c, C57BL/6, and F_1 hybrid mice. A. PFC/spleen. B. PFC/10^6 spleen cells. C. cells/spleen. (r = correlation coefficient). Statistically significant correlation coefficients are indicated: •P = .05.


A
r=-.154
BALB/c
F1
A
r=.765*
C57BL/6
F1
A
r=-.230
B
r=.129
BALB/c
F1
B
r=.878*
C57BL/6
F1
B
r=-.170
C
r=.634
BALB/c
F1
C
r=.833*
C57BL/6
F1
C
r=.460
200
150
100
50
50 100 150 200
C57BL/6
BALB/c
F1
PERCENT CONTROL RESPONSE
PERCENT CONTROL RESPONSE

could suppress the humoral immune response but in these experiments, the mice were treated with carrageenan prior to being immunized. Therefore, an experiment was designed to determine the effect of administering carrageenan at different times (relative to antigen) on the subsequent anti-SRBC response. Table 10 shows the results from two of these experiments. It can be seen that pretreatment with 1 mg carrageenan suppressed the humoral immune response whereas post-immunization treatment had little effect. These data suggest that timing is important in carrageenan-mediated enhancement of the anti-SRBC response.

3.1.5 Effect of Different Types of Carrageenan on the Anti-SRBC Response

As described in the Introduction, there are several molecular species of carrageenans. Since many experiments described in the literature were done using poorly characterized or unfractionated carrageenans, it was important to determine the effect of different types of carrageenans on the primary humoral immune response. Figure 12 shows the effect of kappa-, iota-, and lambda-carrageenan on the anti-SRBC response of BALB/c mice. In terms of their capacity to enhance the immune response, iota- and kappa-carrageenan have similar

TABLE 10. THE EFFECT OF ADMINISTERING KAPPA-CARRAGEENAN AT DIFFERENT TIMES RELATIVE TO ANTIGEN ON THE ANTI-SRBC RESPONSE OF BALB/c MICE

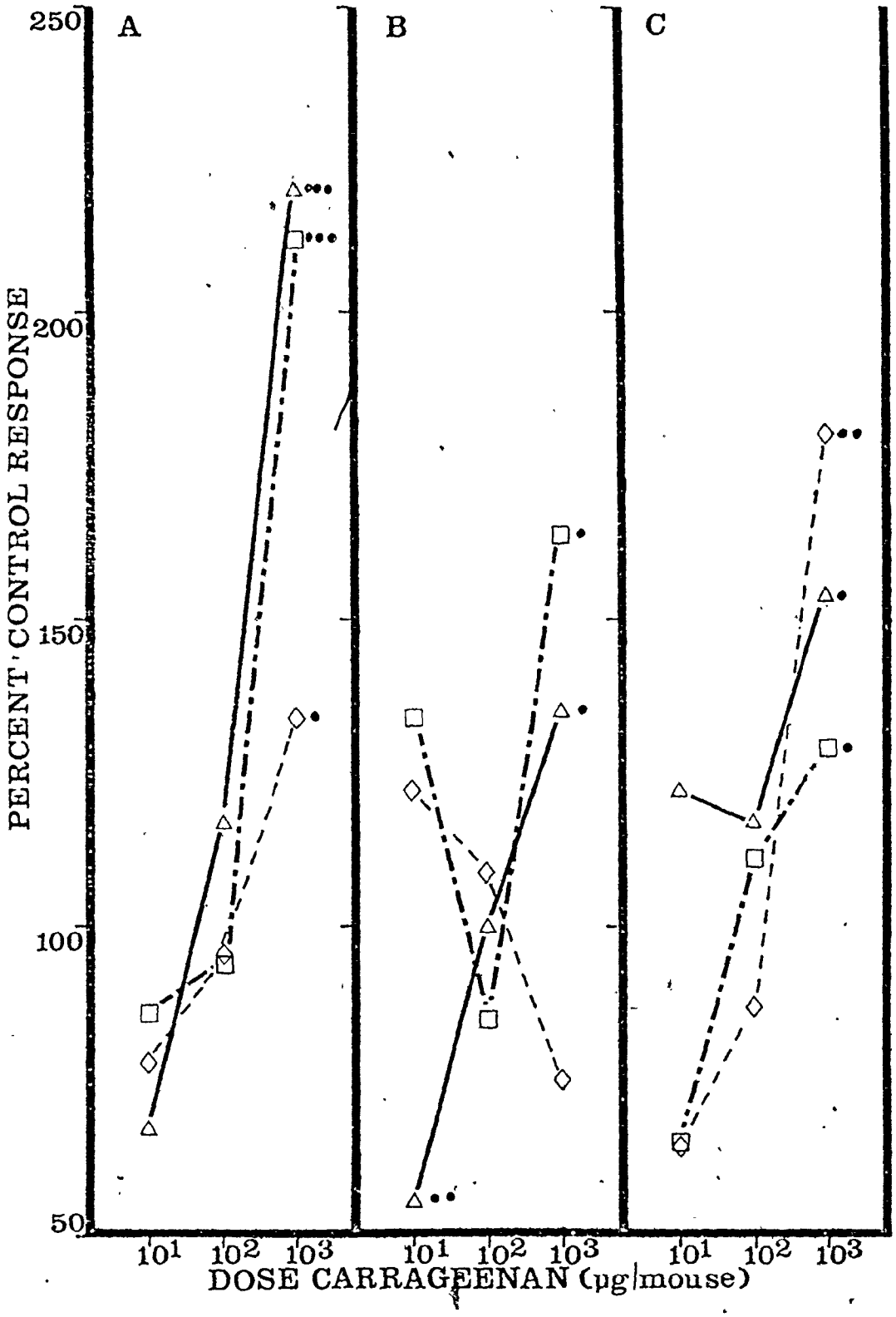
Time of Carrageenan Administration Relative to SRBC (day)(a)	PFC/Spleen (% Control)(b)	
	Expt. 1	Expt. 2
-1	3	2
0	181	N.D.(c)
+1	101	77

(a) All animals (3/treatment group) were injected with $5x10^7$ SRBC on day 0 and 1 mg kappa-carrageenan on the indicated day.

(b) Control animals were injected with $5x10^7$ SRBC only.

(c) A consistent stimulatory effect is seen when kappa-carrageenan is injected on day 0 and so this group was omitted from Expt. 2.

FIGURE 12. The effect of different types of carrageenan on the primary humoral immune response of BALB/c mice. The results are pooled from at least 3 separate experiments for each point (3-6 mice/group/expt.). The mice were injected with the indicated dose and type of carrageenan together with SRBC. Control mice were injected with SRBC only. A. PFC/spleen. B. PFC/10^6 spleen cells. C. cells/spleen Δ——Δ kappa-carrageenan, □---□ iota-carrageenan, O---O lambda-carrageenan.
Significant differences from control values are indicated : ●●●P = .0005, ●●P = .005, ●P = .025.


A
B
C
250
200
150
100
50
PERCENT CONTROL RESPONSE
10^1 10^2 10^3
10^1 10^2 10^3
10^1 10^2 10^3
DOSE CARRAGEENAN (µg/mouse)

effects whereas lambda-carrageenan is considerably less potent and inhibits the number of PFC/10^6 spleen cells. These data suggest that the adjuvant effect of carrageenan has a structural basis.

3.1.6 The Capacity of Carrageenan to Act as a Polyclonal Activator of B Cells

Some experiments were done to determine if carrageenan, like other sulfated polysaccharides, acted as a polyclonal B-cell activator. Figure 13 shows that the administration of carrageenan alone had no effect on the number of background PFC specific for either SRBC or HRBC. Carrageenan treatment augmented the response to SRBC and HRBC. However, when carrageenan was given with antigen it also increased the background PFC response. This is in part explained by carrageenan's capacity to increase the number of cells per spleen but may also be due to some other non-specific stimulatory mechanism.

3.2 *In Vitro* Studies

3.2.1 Tube Culture Experiments

Figure 14 indicates that kappa-carrageenan enhanced

FIGURE 13. The effect of carrageenan treatment on the anti-SRBC and anti-HRBC response of BALB/c mice. The mice (5/group) were injected with red blood cells ($5x10^7$) and/or carrageenan as indicated. The pooled spleens from each treatment group were assayed for anti-SRBC and anti-HRBC PFC five days after treatment. These results represent one experiment. Similar results were obtained in a second experiment. Open bars: anti-SRBC PFC, striped: anti-HRBC PFC, CRG = 1 mg kappa-carrageenan.

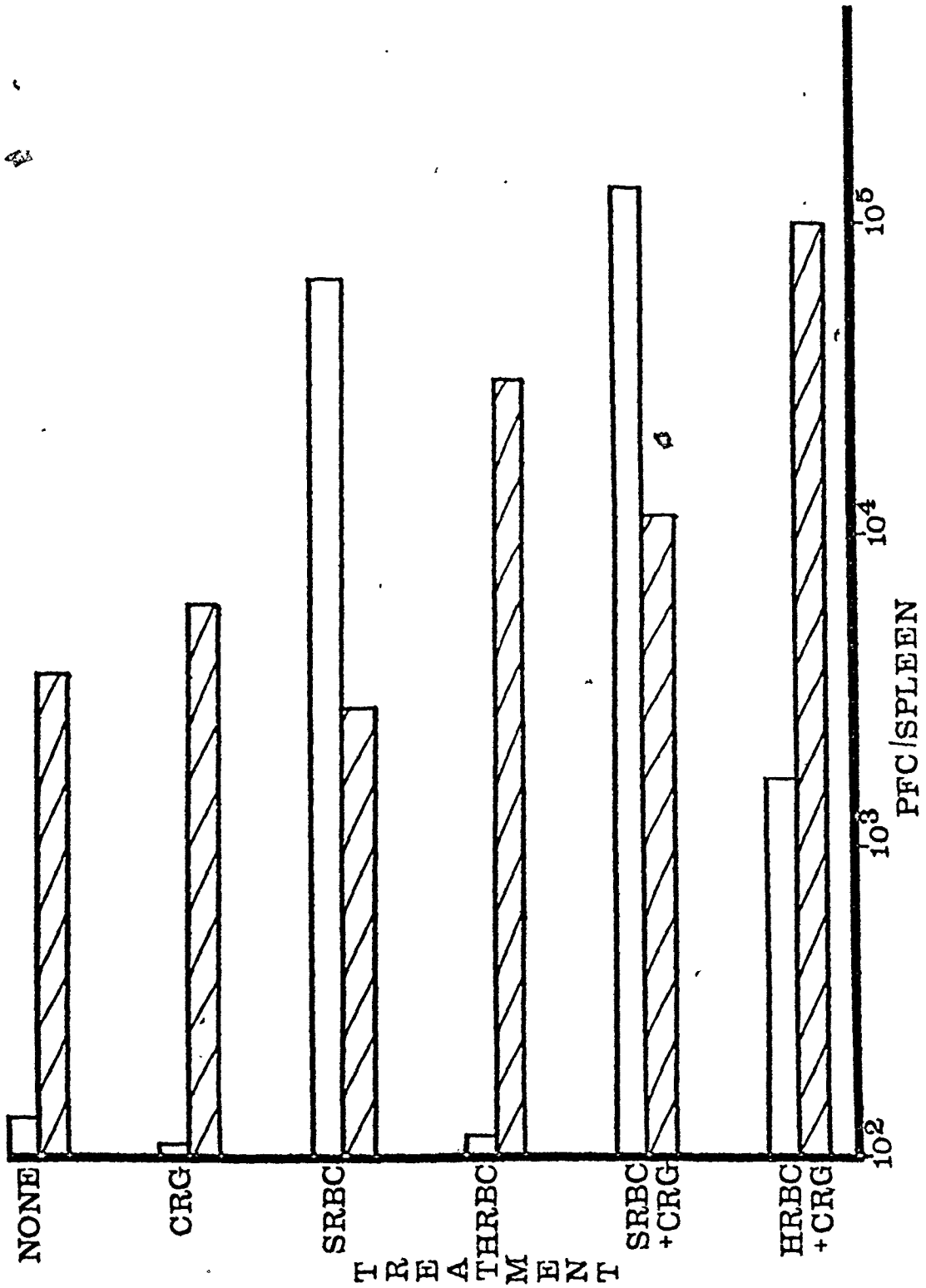
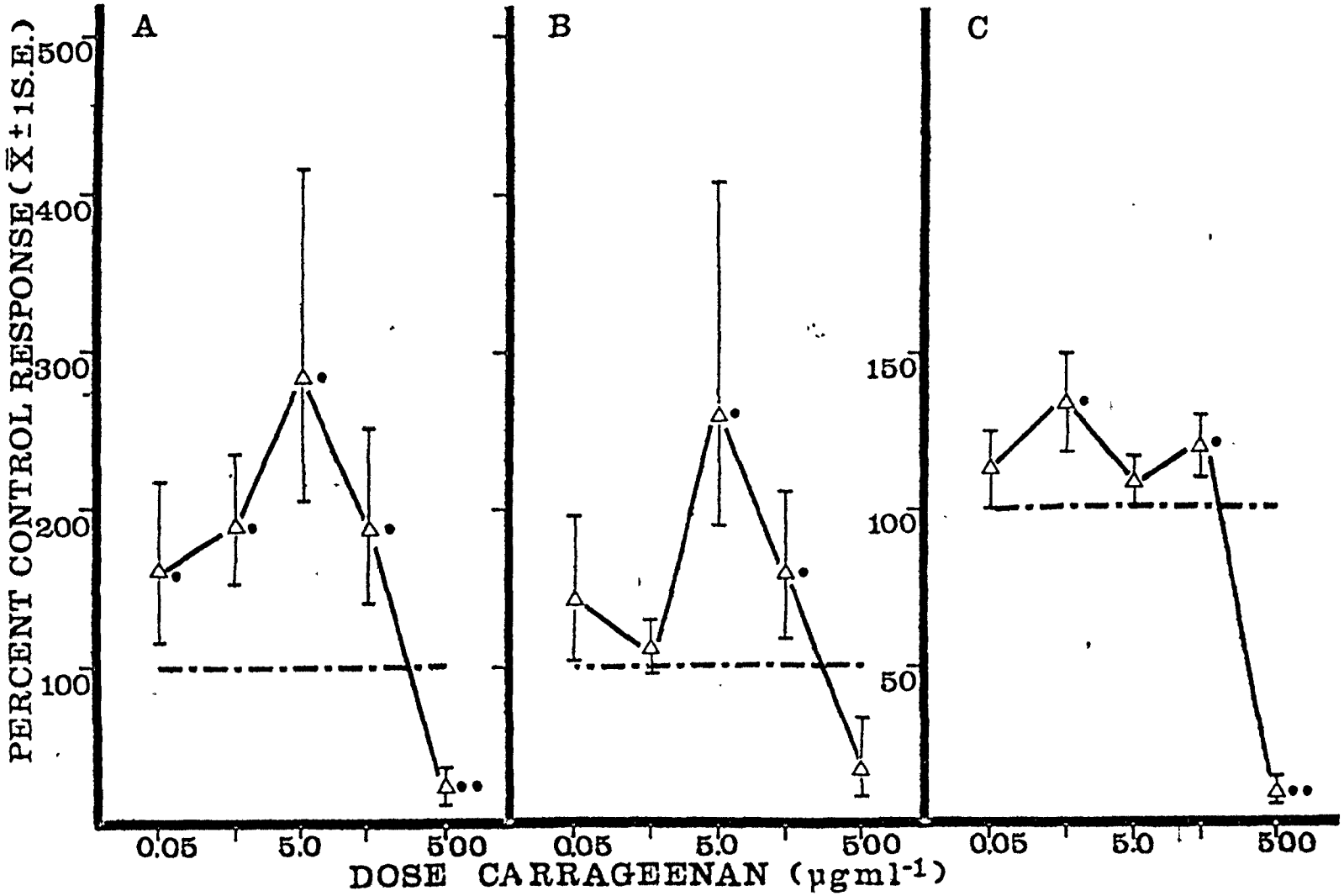

NONE
CRG
SRBC
THRBC
SRBC +CRG
HRBC +CRG
TREATMENT
10^2
10^3
10^4
10^5
PFC/SPLEEN

FIGURE 14. The effect of different doses of kappa-carrageenan on the *in vitro* immune response of BALB/c mice. A. PFC/culture. B. PFC/10^6 spleen cells. C. cells/culture. The indicated dose of kappa-carrageenan and $1x10^6$ SRBC were added to $2x10^6$ spleen cells. The control cultures received SRBC only. The results from two experiments are pooled (n = 7-10 per treatment). Significant differences from controls are indicated: •P = .05, ••P = .005.


A
B
C
PERCENT CONTROL RESPONSE ($\bar{X}$ ± 1S.E.)
500
400
300
200
100
150
100
50
0.05
5.0
500
DOSE CARRAGEENAN (μgml^{-1})

the *in vitro* anti-SRBC response of BALB/c spleen cells with a maximal effect at 5 μg ml^{-1}. This dose of carrageenan did not significantly enhance the number of viable cells/culture although higher and lower doses did. The highest carrageenan dose, 500 μg ml^{-1}, was cytotoxic for BALB/c spleen cells.

For a comparison of the *in vitro* phenomenon with the *in vivo* situation the effect of kappa-carrageenan treatment on the *in vitro* anti-SRBC response of C57BL/6 and F_1 hybrid mice was also examined. For C57BL/6 mice the optimal stimulatory dose was 0.05 μg ml^{-1} although 5.0 μg ml^{-1} also increased the response; 500 μg ml^{-1} kappa-carrageenan was cytotoxic for C57BL/6 spleen cells (figure 15). F_1 hybrid mouse spleen cells like those of C57BL/6 mice were stimulated by 0.05 μg ml^{-1} carrageenan but both 50 and 500 μg ml^{-1} kappa-carrageenan were cytotoxic (figure 16).

As figure 17 shows the *in vitro* responsiveness of these three strains to kappa-carrageenan is different from that found *in vivo* (figure 11). The F_1 hybrid mice responded in a manner which was most similar to C57BL/6 mice (especially in terms of RFC/culture) but all three strains showed a fair degree of correlation with each other compared to what was found *in vivo*. This suggests that the mechanism for carrageenan's direct adjuvant

FIGURE 15. The effect of different doses of kappa-carrageenan on the in vitro immune response of C57BL/6 mice. A. PFC/culture. B. PFC/10^6 spleen cells. C. cells/culture. The indicated dose of kappa-carrageenan and $1x10^6$ SRBC were added to $2x10^6$ spleen cells. The results from two experiments are pooled (n = 7-10 per treatment). Significant differences from controls are indicated: •P = .05, ••P = .025, •••P = .0005.

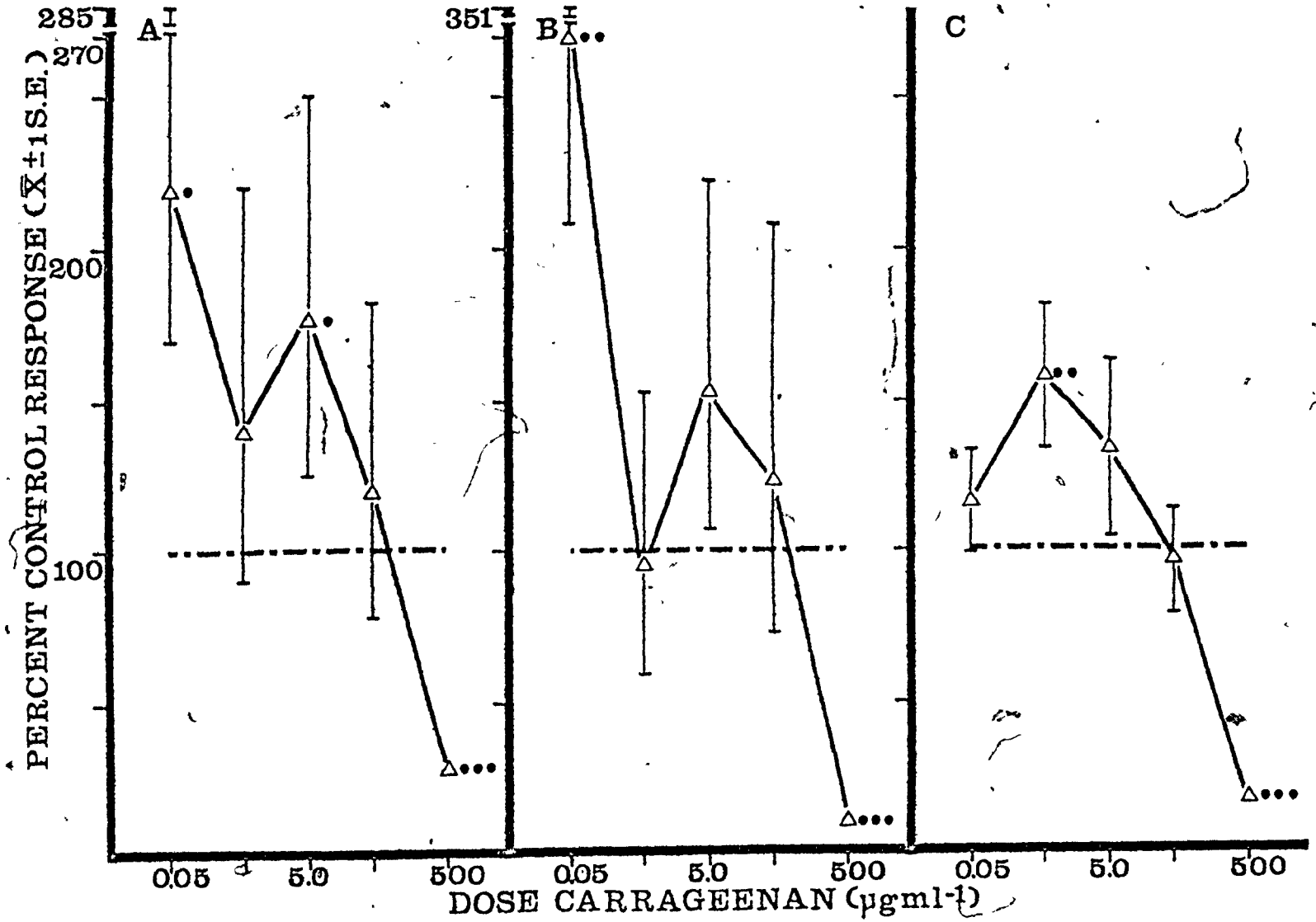

285
270
200
100
A
351
B
C
PERCENT CONTROL RESPONSE (X̄±1S.E.)
0.05
5.0
500
DOSE CARRAGEENAN (μgml-1)

FIGURE 16. The effect of different doses of kappa-carrageenan on the *in vitro* immune response of BALB/c x C57BL/6 F_1 hybrid mice. A. PFC/culture. B. PFC/10^6 spleen cells. C. cells/culture. The indicated dose of kappa-carrageenan and $1x10^6$ SRBC were added to $2x10^6$ spleen cells. The control cultures received SRBC only. The results from three experiments are pooled (n = 9-11 per treatment). Significant differences from controls are indicated: •P = .05, ••P = .025, •••P = .01, ••••P = .0005.

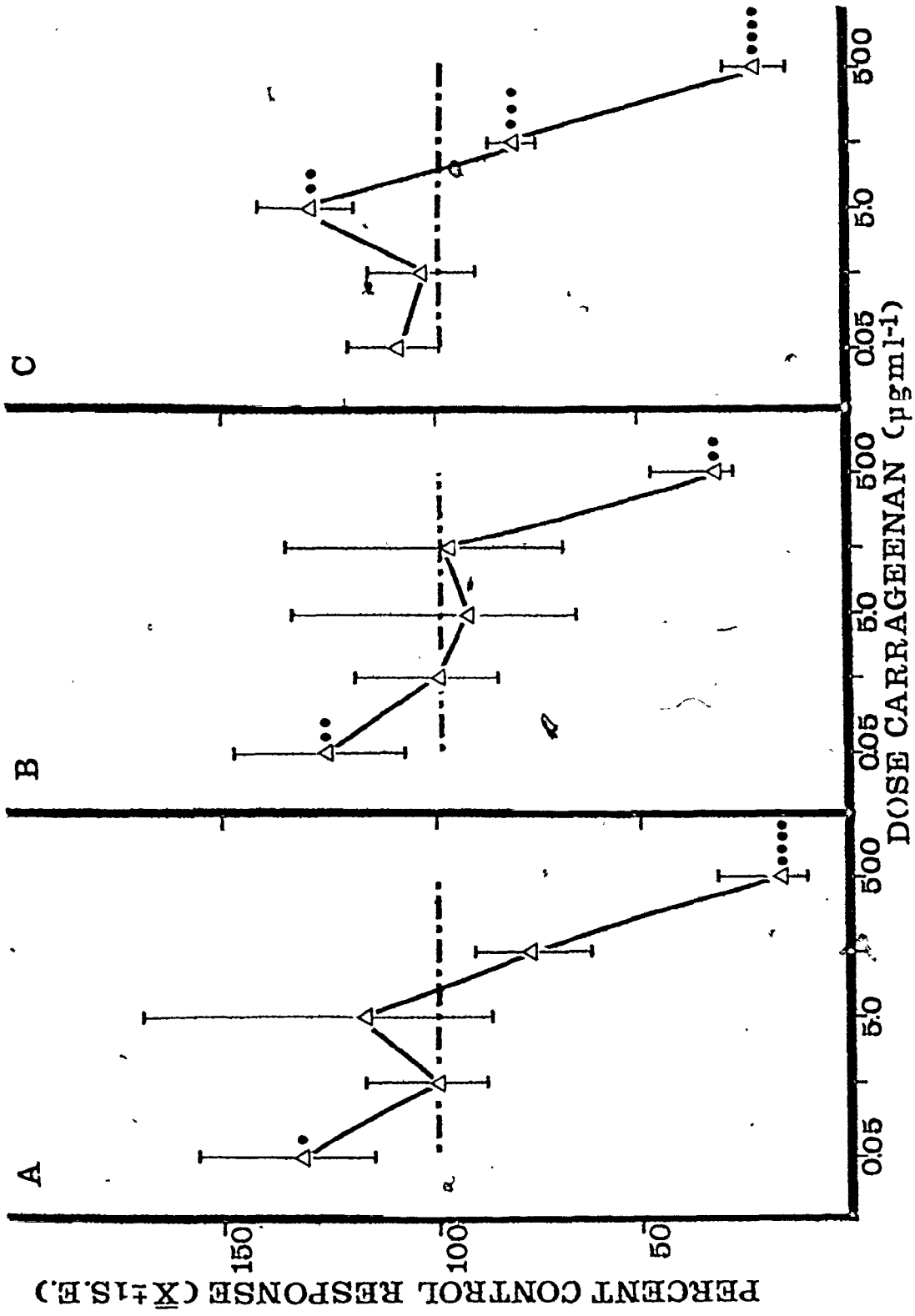
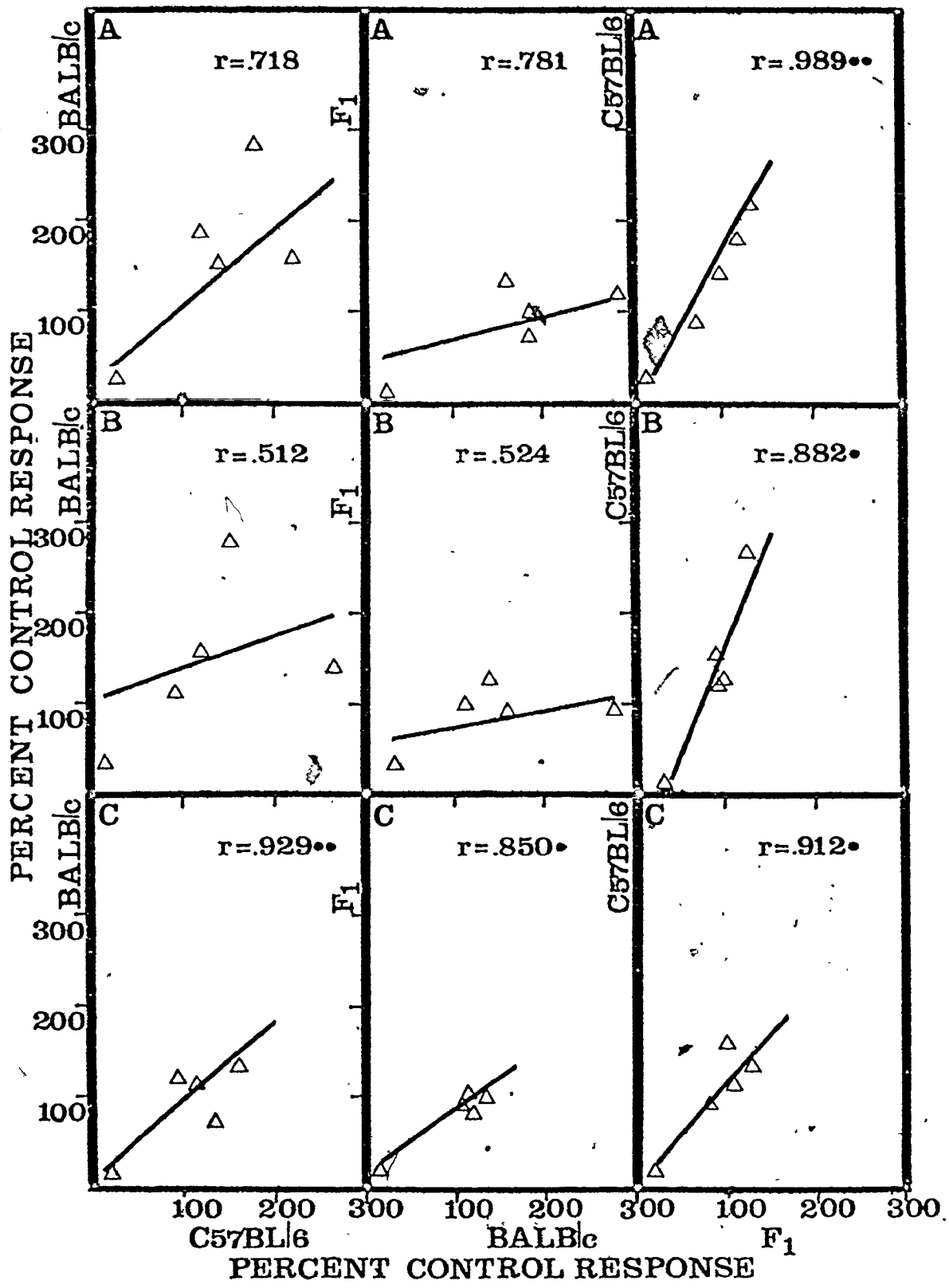

PERCENT CONTROL RESPONSE (X̄±1S.E.)
150
100
50
A
B
C
0.05
0.5
5.0
50.0
DOSE CARRAGEENAN (μgml-1)

FIGURE 17. The relationship between the dose response curves of BALB/c, C57BL/6, and F_1 hybrid mice treated with kappa-carrageenan *in vitro*. A. PFC/culture. B. PFC/10^6 spleen cells. C. cells/culture (r = correlation coefficient). Statistically significant correlation coefficients are indicated: •P = .05.


A
r=.718
BALB/c
F1
300
200
100
A
r=.781
C57BL/6
F1
A
r=.989••
C57BL/6
BALB/c
B
r=.512
B
r=.524
B
r=.882•
C
r=.929••
C
r=.850•
C
r=.912•
100 200 300
C57BL/6
100 200 300
BALB/c
100 200 300
F1
PERCENT CONTROL RESPONSE
PERCENT CONTROL RESPONSE

effect *in vitro* is not the same as the *in vivo* mechanism.

Figure 18 shows the effect of different types of carrageenan on the *in vitro* immune response of BALB/c spleen cells. It can be seen that at a dose level at which kappa-carrageenan enhanced the anti-SRBC response neither iota- nor lambda-carrageenan stimulated it. This is in contrast to the *in vivo* data (figure 12) which showed that iota-carrageenan was about as potent as kappa-carrageenan in enhancing the response. These data suggest that the direct stimulation of the *in vitro* immune response by carrageenans has a structural basis but the structural requirements (in the carrageenan molecule) may differ from those required *in vivo*.

The capacity of kappa-carrageenan to act as a polyclonal activator of B cells *in vitro* is shown in figure 19. Kappa-carrageenan specifically enhanced the PFC response to the administered antigen with no increase in the background PFC response. Thus, kappa-carrageenan at a dose of 5 $\mu g\ ml^{-1}$ does not act as a polyclonal activator of BALB/c B cells.

Because the tube culture experiments suggested that while kappa-carrageenan could act as an adjuvant *in vitro* but that the mechanism of this enhancement may differ from that occurring *in vivo*, a second *in vitro* method was used to assess the *in vivo* mode of action of

FIGURE 18. The effect of different kinds of carrageenan on the in vitro immune response of BALB/c spleen cells. Cultures were set up containing $2x10^6$ spleen cells and the indicated treatments. The dose of carrageenan used was 5 μg ml^{-1} and SRBC were used at a concentration of $1x10^6$ cells per culture. The control cultures were treated with SRBC only. The results from two experiments are pooled (n = 10 cultures per treatment). iCRG = iota-carrageenan, ICRG = lambda-carrageenan.

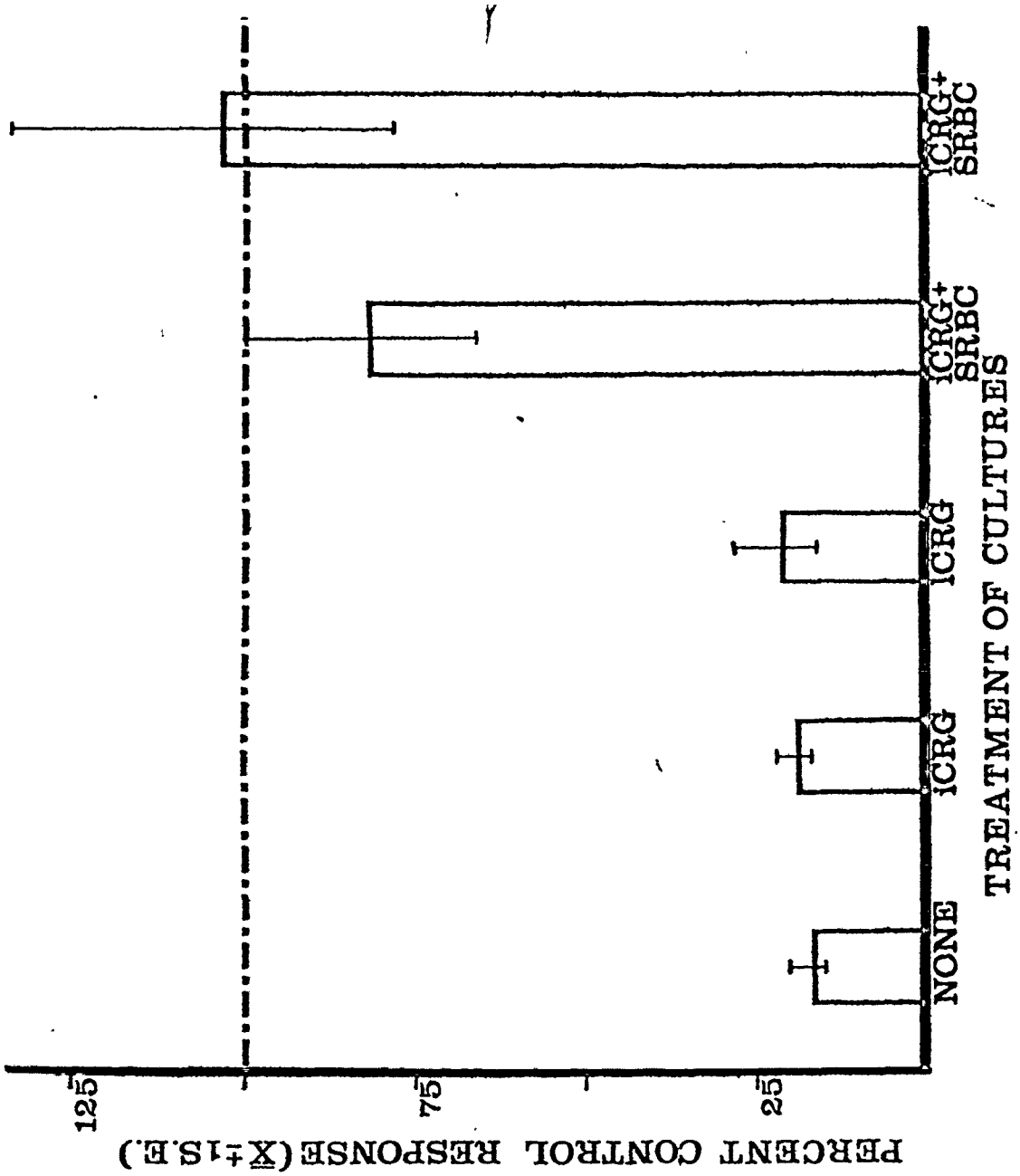
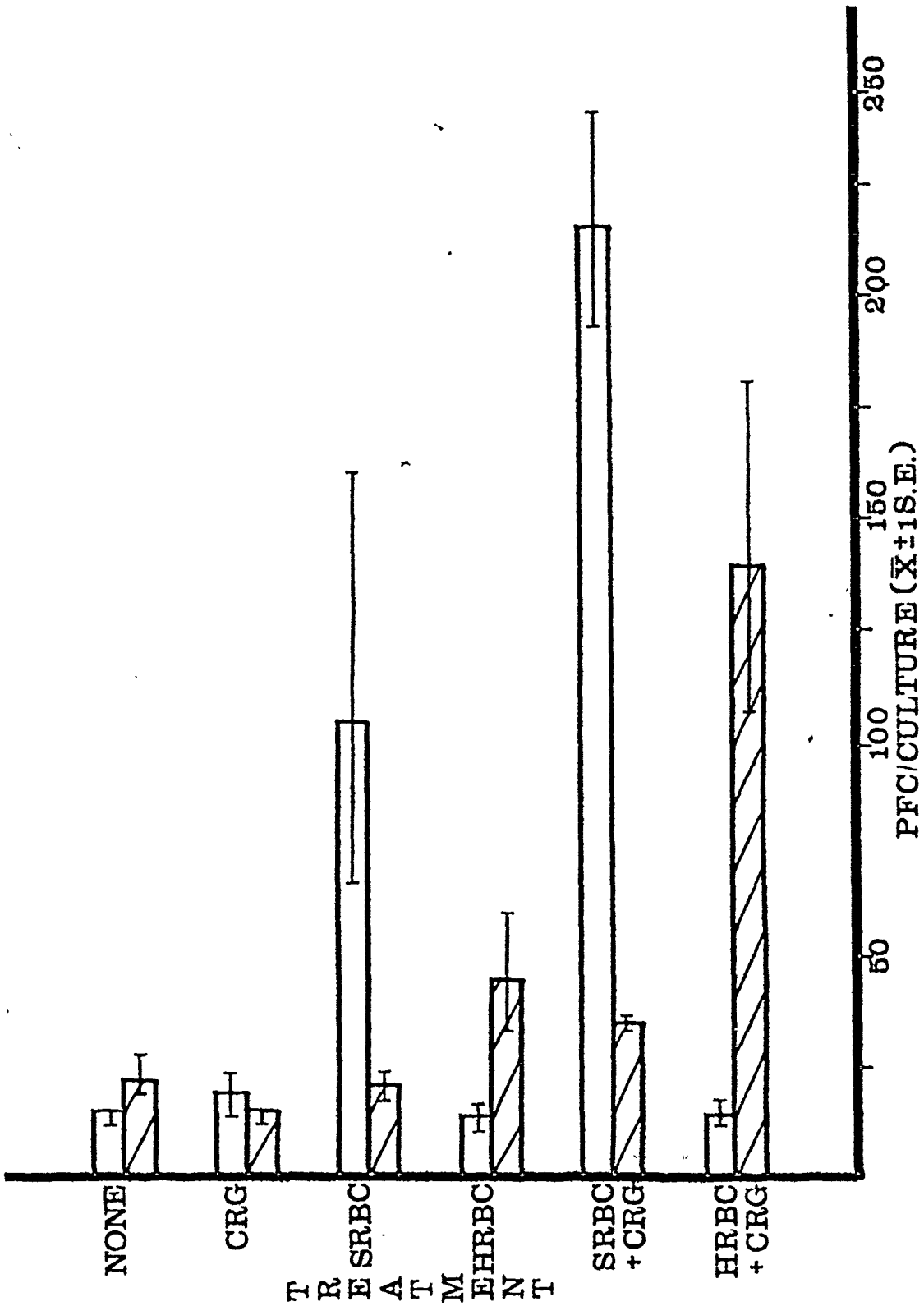

PERCENT CONTROL RESPONSE ($\bar{X}$±1S.E.)
125
75
25
NONE
ICRG
ICRG
ICRG+ SRBC
ICRG+ SRBC
TREATMENT OF CULTURES

FIGURE 19. The effect of kappa-carrageenan treatment on the *in vitro* immune response of BALB/c spleen cells to SRBC and HRBC. Each culture consisted of $2x10^6$ spleen cells plus the indicated treatment (5 $\mu g\ ml^{-1}$ kappa-carrageenan, $1x10^6$ red blood cells/culture). The open bars indicate anti-SRBC PFC and the striped bars show anti-HRBC PFC. There were five replicates per treatment. The results of one experiment are presented. A second experiment gave similar results. CRG = carrageenan.


PFC/CULTURE ($\bar{X}$±1S.E.)
50
100
150
200
250
NONE
CRG
SRBC
EHRBC
SRBC +CRG
HRBC +CRG
TREATMENT

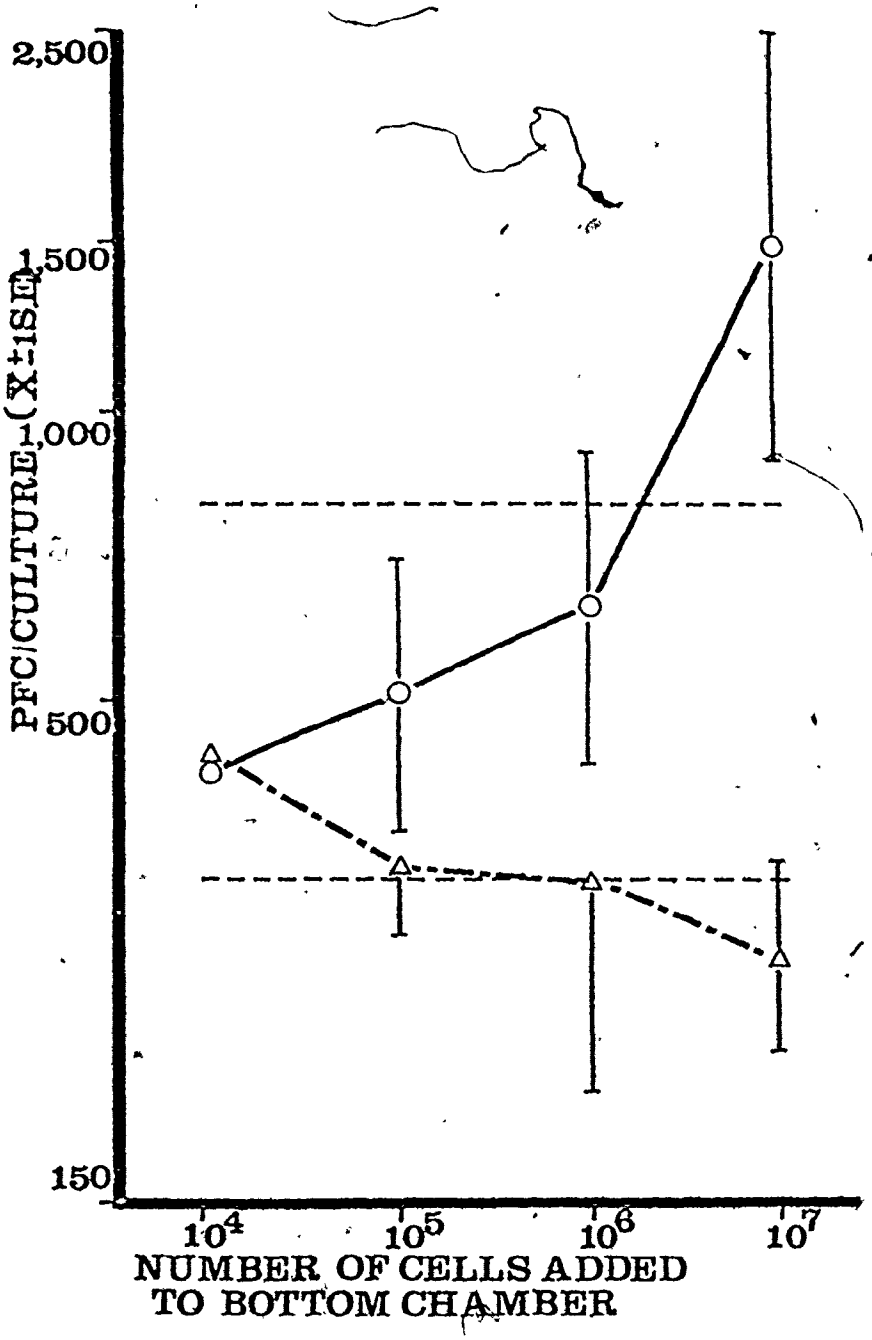
carrageenan as an adjuvant.

3.2.2 Marbrook Vessel Experiments

The effect of different numbers of carrageenan-treated spleen cells on the PFC response in the top chamber of Marbrook vessels is shown in figure 20. See section 2.2.4 for a description of Marbrook vessels. Untreated cells, or medium alone did not affect the response in the top chamber. In contrast, carrageenan-treated cells caused a dose-dependent enhancement of the response in the top chamber. This enhancement must have been due to the release of a soluble factor(s) by the carrageenan-treated cells since the two chambers were separated by a dialysis membrane. When 10^7 carrageenan-treated cells were placed in the bottom chamber but there was no antigen in the top chamber the response of the top chamber cells was not significantly above background levels. These data show that carrageenan-treated cells can release dialysable soluble factor(s) which enhance the PFC response in the presence of antigen.

In order to determine the relationship between carrageenan treatment and enhancement of the top chamber PFC response, spleen cells from mice injected with different doses of kappa-carrageenan were placed in the

FIGURE 20. The effect of different numbers of either normal spleen cells (Δ----Δ) or carrageenan-treated spleen cells (O——O) in the bottom chamber on the number of PFC developing in the top chamber. The top chamber contained $10x10^6$ normal spleen cells and $5x10^6$ SRBC. The area bordered by the horizontal dashed lines indicates the response of the top chamber cells when the bottom chamber contained only medium. The results are pooled from four separate experiments except for $1x10^4$ cells in the bottom chamber which was done in only one experiment. Carrageenan-treated spleen cells came from BALB/c mice which had been injected with 1 mg kappa-carrageenan 6 hr prior to setting up the Marbrook vessel.


2,500
1,500
1,000
500
150
PFC/CULTURE (X±1SE)
10^4
10^5
10^6
10^7
NUMBER OF CELLS ADDED
TO BOTTOM CHAMBER

bottom chamber (figure 21). It can be seen that the amount of enhancement mediated by carrageenan-treated cells is related to the dose of carrageenan the spleen cell donors received *in vivo*. A comparison between these results and the *in vivo* effect of the different doses of carrageenan reveals a statistically significant correlation (figure 22) suggesting that the *in vitro* phenomenon (release of soluble enhancing factor(s)) is related to the *in vivo* phenomenon (modulation of the immune response).

Due to a limitation in the number of Marbrook vessels, not all experiments included both medium only and normal cell controls. Table 11 shows that in the 12 experiments where medium and normal cell controls were both used the response in the top chamber was virtually identical. In later experiments either normal cells or medium alone were used as controls.

Enhancement of the response of top chamber cells to SRBC by supernatants produced by carrageenan-treated cells is shown in figure 23 adding further evidence to the argument that the enhancement of the top chamber response by carrageenan-treated cells is mediated by soluble factor(s). Supernatants produced by untreated cells boosted the response in the top chamber above that observed when medium alone was present in the bottom

FIGURE 21. The effect of spleen cells from mice injected with different doses of carrageenan on the anti-SRBC response of top chamber cells. Spleen cells (10^7) from BALB/c mice injected with the indicated dose of kappa-carrageenan were placed in the bottom chamber of Marbrook vessels. The top chamber contained 10^7 normal spleen cells together with 5×10^6 SRBC. There were five Marbrook vessels per treatment. Cultures containing spleen cells from mice injected with 1 mg carrageenan gave significantly higher responses ($P = .025$) than cultures containing untreated spleen cells.

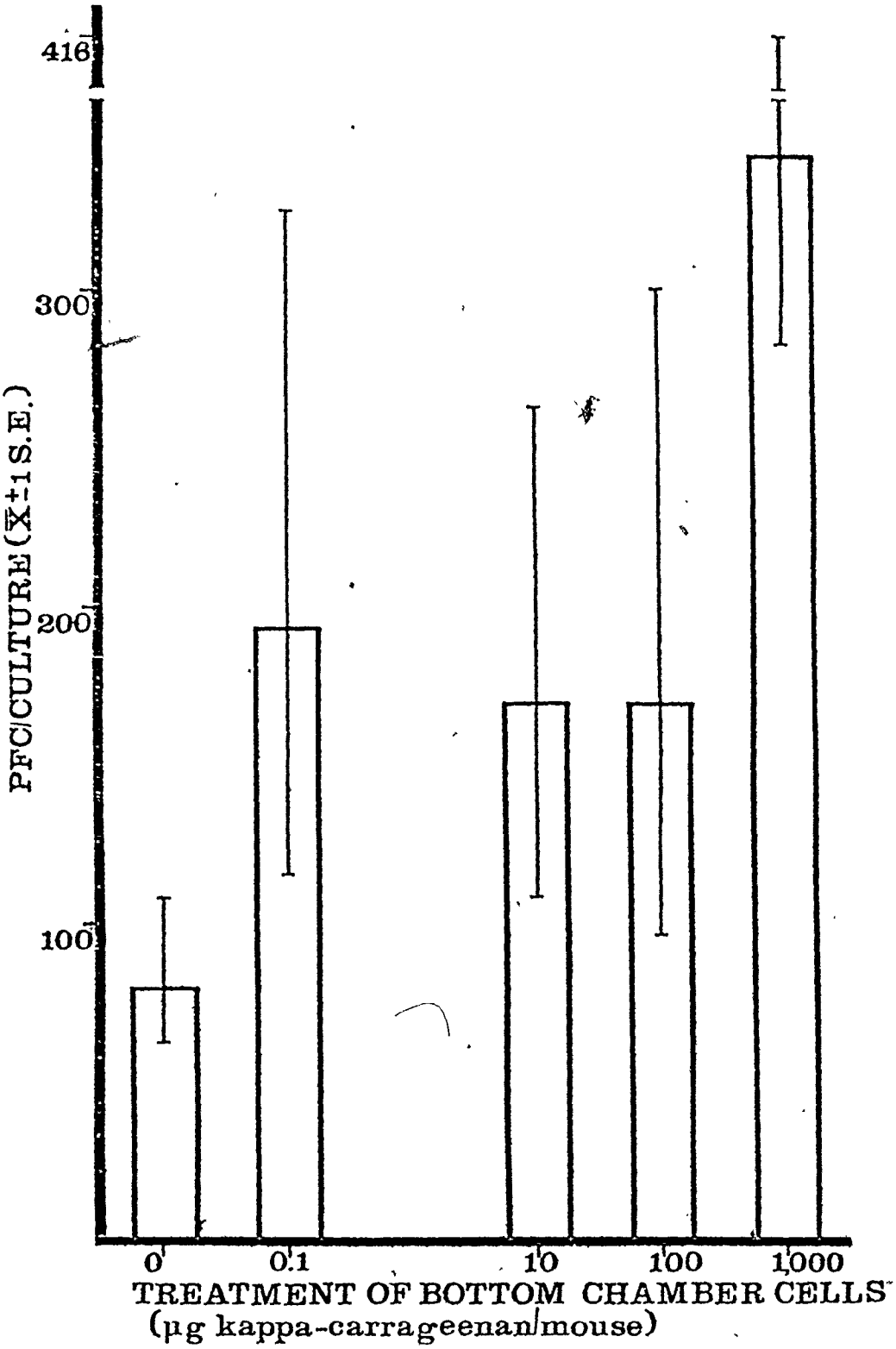

416
300
200
100
PFC/CULTURE ($\bar{X}$±1 S.E.)
0
0.1
10
100
1,000
TREATMENT OF BOTTOM CHAMBER CELLS
(µg kappa-carrageenan/mouse)

FIGURE 22. The relationship between *in vivo* modulation of the immune response by kappa-carrageenan and the *in vitro* release of soluble enhancing factor(s) by carrageenan-treated cells. Spearman's coefficient of rank correlation of these two curves was .950 (P = .05).

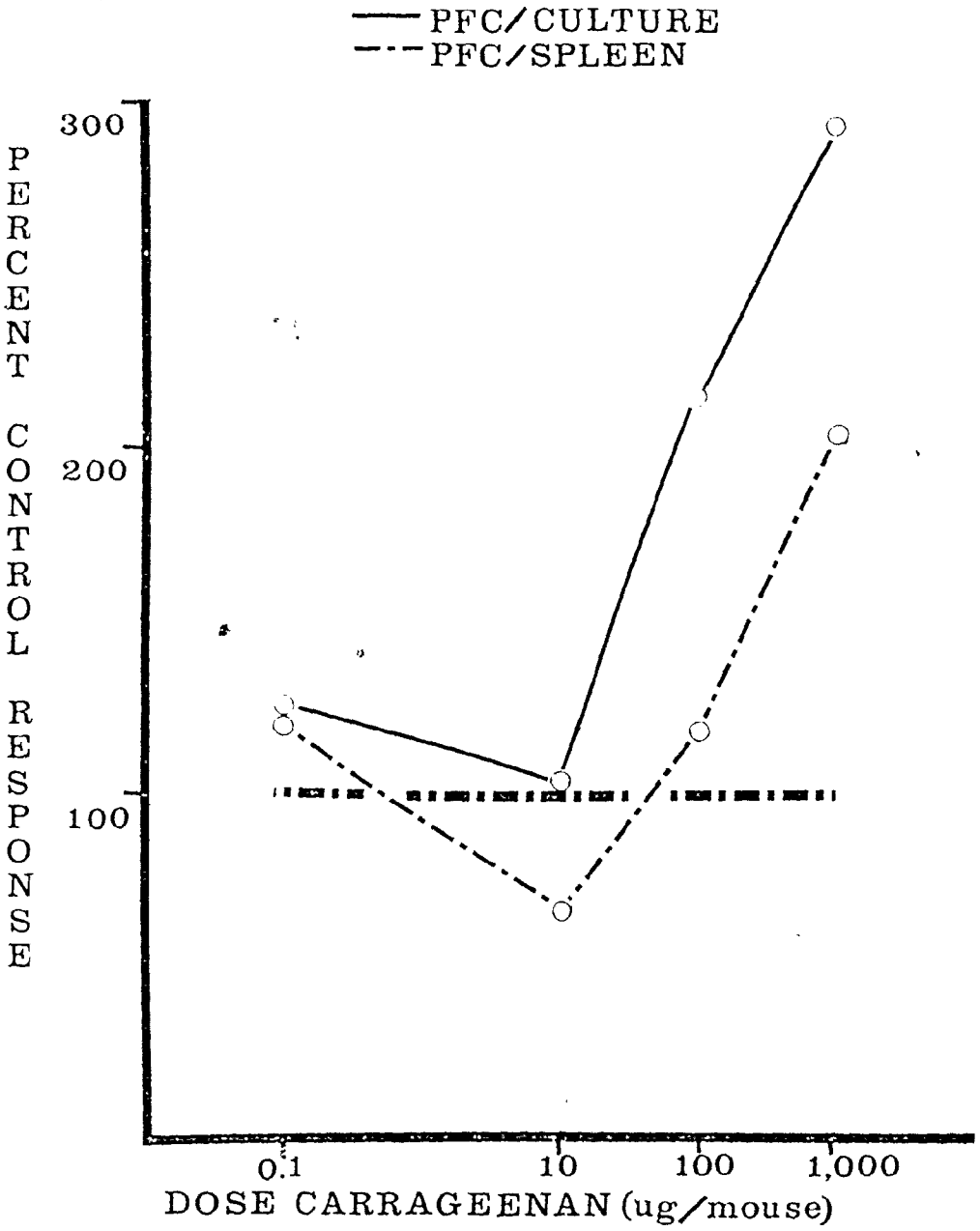

PFC/CULTURE
PFC/SPLEEN
PERCENT CONTROL RESPONSE
300
200
100
0.1
10
100
1,000
DOSE CARRAGEENAN (ug/mouse)

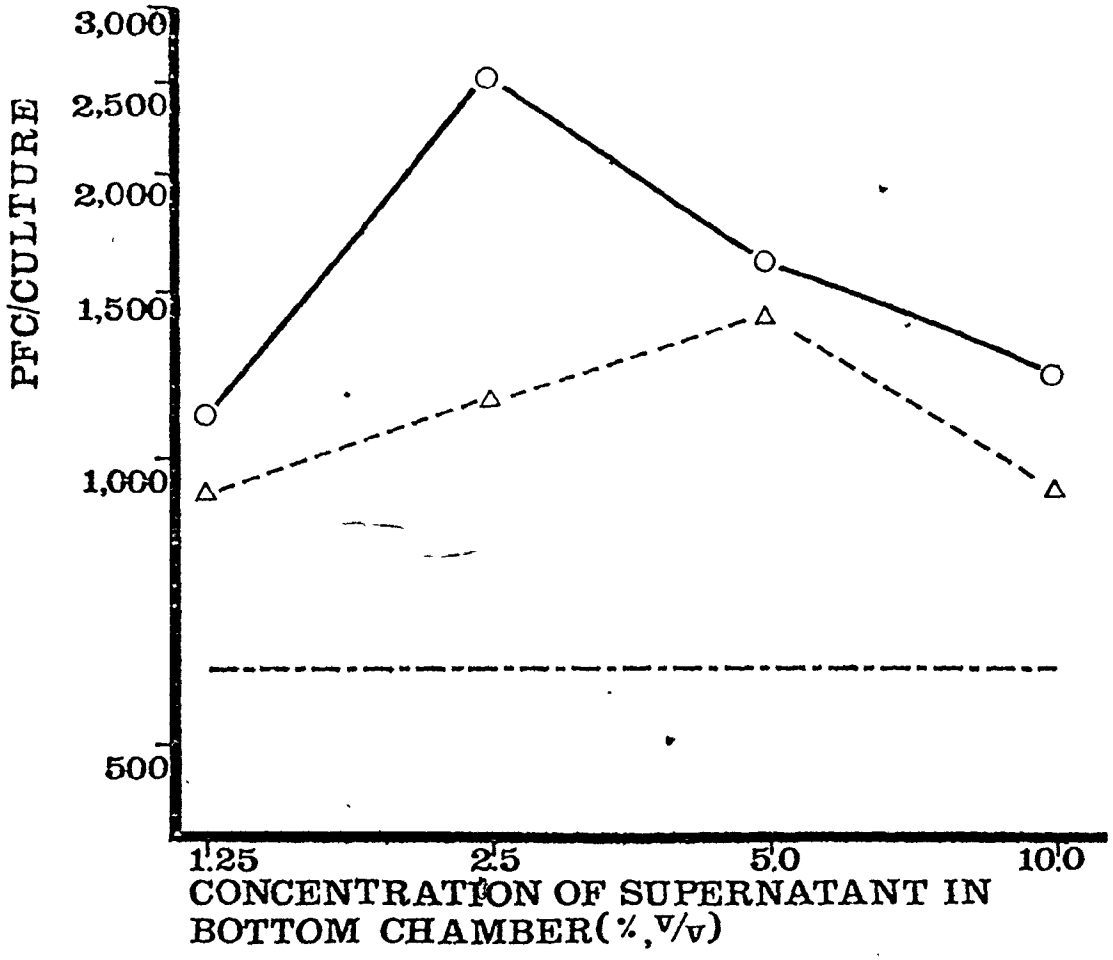
TABLE 11. A COMPARISON OF MEDIUM CONTROLS AND NORMAL CELL CONTROLS

Bottom Chamber Treatment	$\bar{X} \pm 1$ S.E. (b)		
	PFC/Culture(a)	PFC/10^6 Spleen Cells	Cells/Culture x 10^6
medium	1,364(1,080-1,649)	628(456-799)	2.75(2.38-3.12)
$10x10^6$ normal cells	1,327(966-1,688)	632(480-785)	2.36(1.92-2.80)

(a) $10x10^6$ normal cells in top chamber + $5x10^6$ SRBC

(b) These results are pooled from 12 experiments

FIGURE 23. The effect of supernatants on the number of PFC developing in the top chamber. Supernatants were prepared by incubating either carrageenan-treated cells (10×10^6 in 10 mls basic medium with 10% fetal calf serum) ●——● or normal cells (same conditions as above) ▲----▲ for three days. The cultures were then centrifuged (1,000 g for 8 min.) and the supernatant fluid was added to the bottom of Marbrook vessels. The effect of adding no supernatant to the bottom chamber is indicated (—— - ——). The results are pooled from two separate experiments.


3,000
2,500
2,000
1,500
1,000
500
PFC/CULTURE
1.25
2.5
5.0
10.0
CONCENTRATION OF SUPERNATANT IN BOTTOM CHAMBER(%,V/V)

chamber possibly because some additional fetal calf serum (used to culture the supernatant-producing cells) was added to the bottom chamber in the supernatants. These supernatants were also found to stimulate the anti-SRBC response of tube cultures.

The optimal immunogenic dose for top chamber spleen cells was found to be $5x10^6$ SRBC (figure 24). Although carrageenan-treated cells enhanced the response of top chamber cells at all three SRBC doses, the amount of enhancement was at a maximum when the response in the top chamber was at its minimum. The response of both the carrageenan-treated and the untreated spleen cells in the bottom chamber was at a background level (figure 24). Therefore it cannot be argued that the release of soluble factor(s) by carrageenan-treated cells depends on an anti-SRBC response in the bottom chamber.

The effect of carrageenan-treated cells on two types of responses in the top chamber is shown in table 12. The number of anti-SRBC PFC in the top chamber was clearly enhanced when the response in that chamber was "low", but was not affected when the response was "high", suggesting that in order for the soluble enhancing factor(s) to stimulate the anti-SRBC response in the top chamber the factor(s) must be in demand. Since the magnitude of enhancement by carrageenan-treated cells

FIGURE 24. The effect of different numbers of SRBC in the top chamber on the number of PFC. The indicated number of SRBC were placed in the top chamber together with $10x10^6$ normal spleen cells. The bottom chamber contained either $10x10^6$ normal spleen cells (open bars) or $10x10^6$ carrageenan-treated spleen cells (striped bars). The PFC response of the bottom chamber cells was also measured (Δ - normal cells, O - carrageenan-treated cells). Standard errors (not shown) were approximately 20% of the mean.

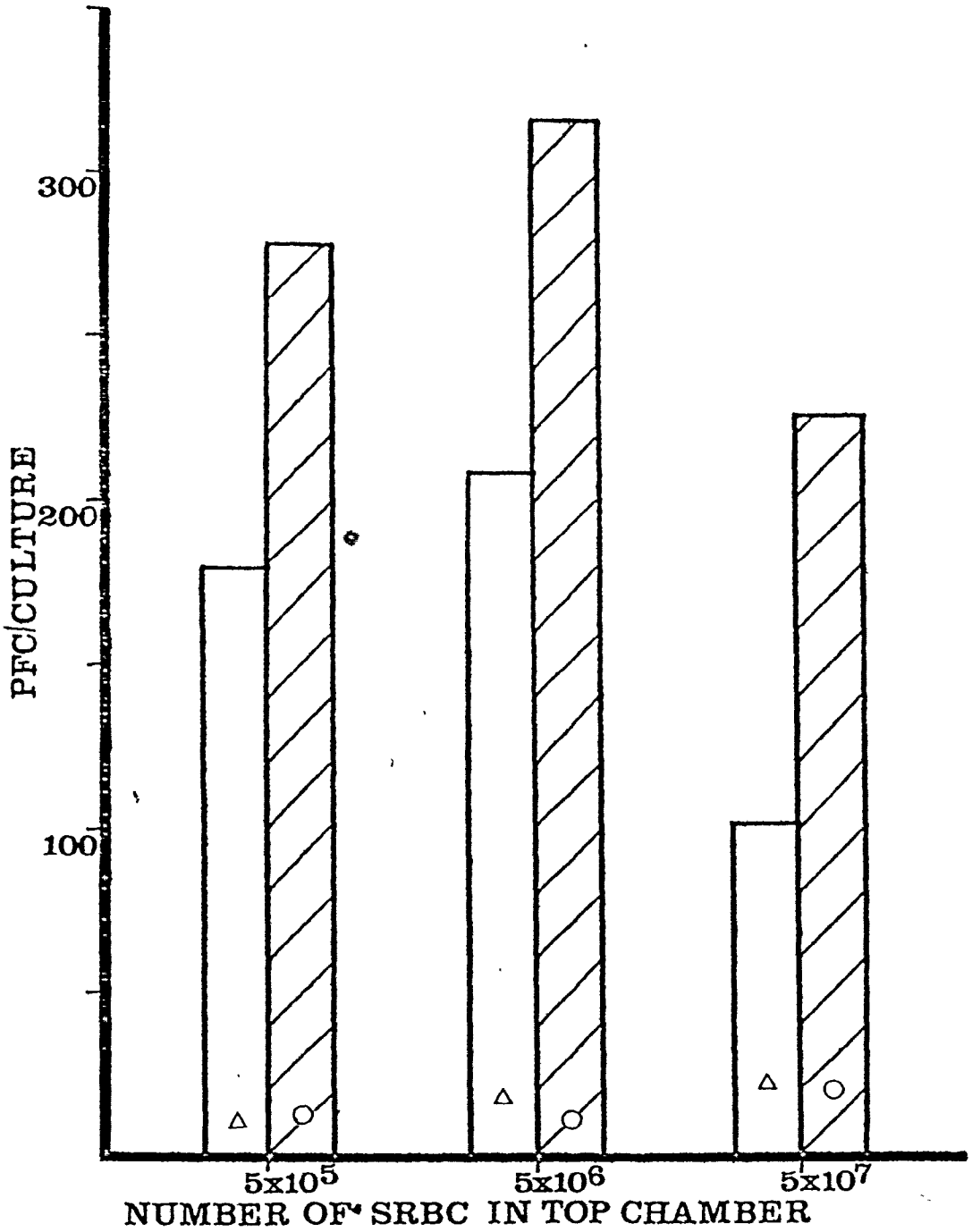

300
200
100
PFC/CULTURE
△
○
5x10^5
5x10^6
5x10^7
NUMBER OF SRBC IN TOP CHAMBER

TABLE 12. THE EFFECT OF CARRAGEENAN-TREATED CELLS ON "HIGH" AND "LOW" RESPONSES IN MARBROOK VESSELS

Type of Top Chamber Response	Bottom Chamber Cells	$\bar{X} \pm 1$ S.E. (a)		
		PFC/Culture	PFC/10^6 Cells	Cells/Culture ($\times 10^{-6}$)
low (b)	normal	880(636-1125)	499(376-621)	2.11(1.77-2.44)
	carrageenan	2,009(1,595-2424)	794(615-973)	3.24(2.74-3.73)
high (c)	normal	2,745(2,215-3276)	906(635-1177)	3.70(2.96-4.44)
	carrageenan	2,521(1,907-3134)	940(567-1313)	3.32(2.83-3.82)

(a) 10×10^6 cells in top chamber + 5×10^6 SRBC

(b) Results are pooled from 11 experiments

(c) Results are pooled from 5 experiments

Note: The "high" and "low" results came from separate experiments

was to the level obtained with "high" responding untreated cells the soluble enhancing factor(s) may be a normal component of the immune response.

Reducing the number of cells in the top chamber, which reduced the anti-SRBC response, enhanced the effect induced by the carrageenan-treated cells (figure 25). In this experiment the response in the top chamber was "high". This might indicate that the soluble factor(s) produced by carrageenan-treated cells became limiting (in the top chamber) and so the carrageenan-treated cells in the bottom chamber could produce an enhancing effect.

Table 13 shows the effect of fractionated bottom chamber cells on the anti-SRBC response in the top chamber. It can be seen that fractionation of normal cells had little effect on the response in the top chamber (II and III vs I). Carrageenan-treated cells enhanced the anti-SRBC response in the top chamber (IV vs I) but the removal of T cells from the carrageenan-treated cells reduced the response in the top chamber to the level observed when normal spleen cells were in the bottom chamber (V vs I). The removal of adherent cells on the other hand had little effect on the amount of enhancement produced by carrageenan-treated spleen cells (VI vs I). These data suggest that the soluble

FIGURE 25. The effect of decreasing the number of cells in the top chamber on the magnitude of the carrageenan-induced enhancement. The top chamber contained 5×10^6 SRBC together with the indicated number of normal spleen cells. The bottom chamber contained either 10×10^6 carrageenan-treated cells (o----o) or 10×10^6 normal cells (Δ----Δ). The results are expressed as the geometric mean of two cultures at each point. A second experiment gave similar results.

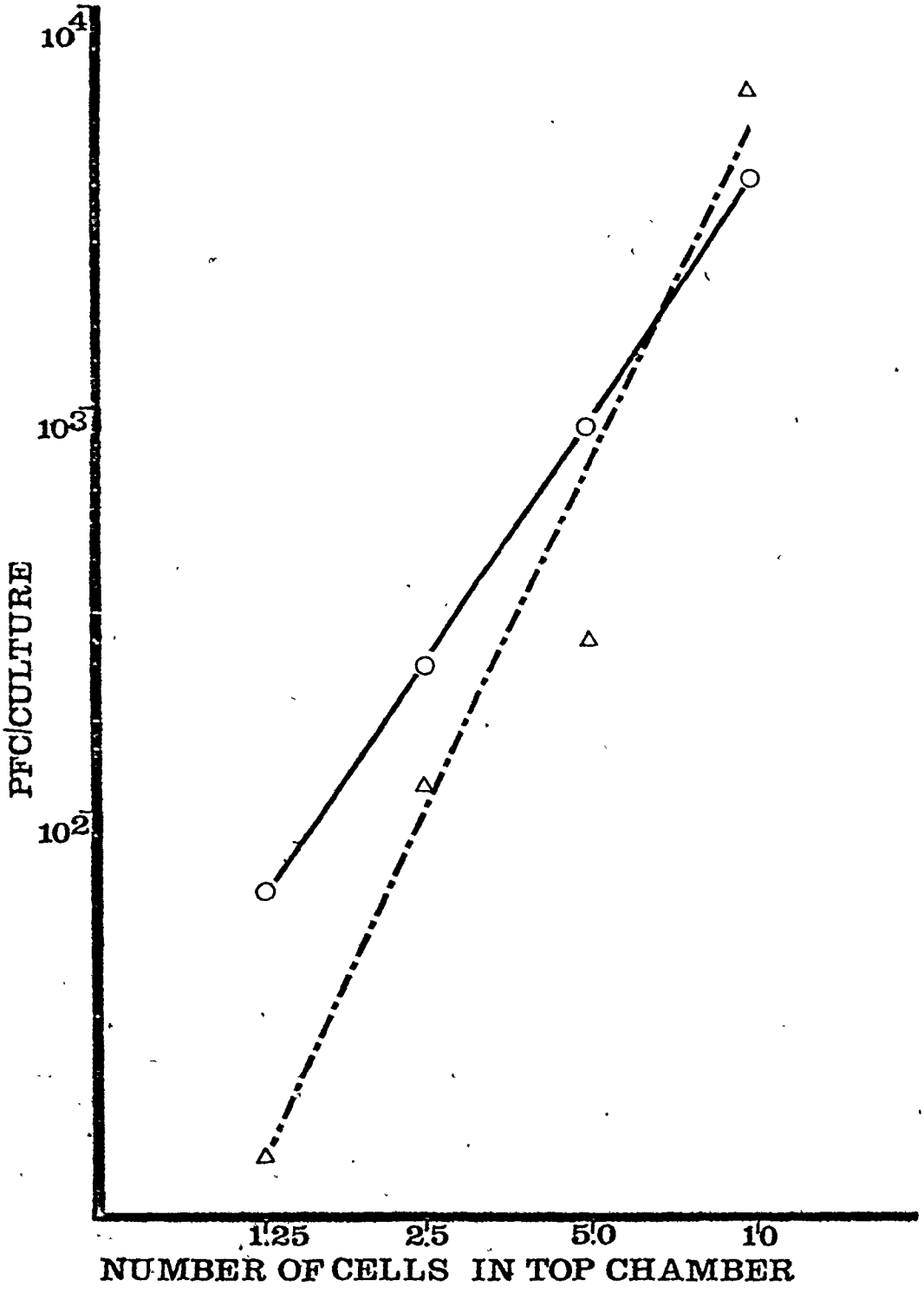

10^4
10^3
10^2
PFC/CULTURE
1.25
2.5
5.0
10
NUMBER OF CELLS IN TOP CHAMBER

TABLE 13. THE CELLULAR BASIS FOR THE PRODUCTION OF THE SOLUBLE ENHANCING FACTOR(S). THE TOP CHAMBER CONTAINED 5×10^7 SRBC PLUS 10×10^6 NORMAL SPLEEN CELLS. FIVE CULTURES WERE ESTABLISHED FOR EACH TREATMENT

Treatment Group	Bottom Chamber Cells		No. PFC Developing in Top Chamber ($\bar{X} \pm 1$ S.E.)
	Source (a)	Treatment	
I	normal	none (b)	458(367-571)
II	normal	anti-thy 1.2 (c)	446(265-751)
III	normal	carbonyl iron (d)	331(146-750)
IV	carrageenan	none	821(792-850)
V	carrageenan	anti-thy 1.2	451(383-531)
VI	carrageenan	carbonyl iron	869(840-900)

(a) Injected with 1 mg kappa-carrageenan 6-12 hours prior to culturing or untreated

(b) 10^7 cells/culture

(c) Non-T cells; 5×10^6 cells/culture

(d) Non-adherent cells; 1×10^6 cells/culture

NOTE: Anti-thy 1.2 serum showed effective elimination of thy 1^+ cells at dilutions of up to 1:500, killing approximately 50% of splenic lymphocytes. Nude mouse spleen cells were unaffected by treatment with anti-thy 1.2 + complement.

enhancing factor(s) is produced by a nonadherent T cell.

Recombining the fractionated cells, non-adherent carrageenan-treated spleen cells and normal non-T cells enhanced the PFC response in the top chamber (table 14). This is in agreement with table 13 since the carrageenan-treated T cells were present in this mixture and it suggests that normal non-T cells do not inhibit the production of the soluble enhancing factor(s). Group IV, consisting of carrageenan-treated non-T cells and normal non-adherent cells, enhanced the anti-SRBC response in the top chamber (IV vs I). Table 13 shows that when used alone neither of these populations boosted the response in the top chamber. These data suggest that cell-cell interaction may also be involved in the generation of soluble enhancing factor(s).

TABLE 14. CELLULAR INTERACTIONS INVOLVED IN GENERATING SOLUBLE ENHANCING FACTORS. THE TOP CHAMBER CONTAINED $5x10^6$ SRBC TOGETHER WITH $10x10^6$ NORMAL SPLEEN CELLS. FIVE CULTURES WERE ESTABLISHED FOR EACH TREATMENT.

Treatment Group	Cells Added to Bottom Chamber	No. PFC Developing in Top Chamber ($\bar{X}$ ± 1 S.E.)
I	normal spleen (a)	944(830-1075)
II	carrageenan spleen (b)	1698(1192-2412)
III	non-adherent carrageenan spleen (c) + normal non-T cells (d)	1771(1488-2108)
IV	carrageenan non-T cells (d) + non-adherent normal spleen (c)	1848(1658-2011)

(a) 10^7 cells/culture

(b) From mice injected with 1 mg kappa-carrageenan, 10^7 cells/culture

(c) $1x10^6$ cells/culture

(d) $5x10^6$ cells/culture

Chapter 4

DISCUSSION

4.1 In Vivo Studies

The results presented in this thesis indicate that kappa-carrageenan acts as an adjuvant for the primary humoral immune response of BALB/c mice (figure 8, tables 8 and 9). This adjuvant effect is expressed in terms of both IgM- and IgG-producing cells (table 9).

The dose of kappa-carrageenan (1 mg) which augmented the anti-SRBC response of BALB/c mice also increased the number of cells per spleen (figure 8c). Others have also reported that carrageenan increases the number of cells per spleen (Thomson et al., 1976; Quan et al., 1980). Dextran and pentosan sulfate also increase spleen cellularity by delaying the rate of progress of lymphocytes through the marginal zone of the spleen (Freitas and De Sousa, 1977) and it is possible that the increase in spleen cell number caused by carrageenan is produced by a similar mechanism.

Since this increase in spleen cell number was also seen in C57BL/6 mice without a concomitant adjuvant effect it is unlikely that this process defines carrageenan's mode of action as an adjuvant.

The effect of carrageenan treatment on the anti-SRBC response of BALB/c mice clearly depends on the dose of carrageenan (figure 8). The dose-response curve of BALB/c mice to kappa-carrageenan had a bimodal distribution. Low doses of carrageenan caused a small increase in the anti-SRBC response, intermediate doses suppressed the response, and high doses significantly elevated it. Pawlec and Brons (1978) observed a bimodal dose-response curve when they examined the effect of lambda-carrageenan on the mixed-lymphocyte reaction and the dose-response curve of mouse lymphocytes to concanavalin A can also assume a bimodal shape (Naidorf et al., 1980). Since both of these responses are T-cell responses, it seems that agents which act on these cells can suppress or enhance immune reactions in a dose-dependent manner. Most adjuvants show a unimodal dose-response curve with low doses having no effect on the response, an intermediate dose-range being stimulatory, and high doses acting suppressively. High dose of kappa-carrageenan (4 mg) were also immuno-suppressive which may represent a toxic effect as is the

situation with other immunostimulants. The inhibition of the immune response by intermediate doses of kappa-carrageenan was probably related to its target cell. If, as I shall argue later, carrageenan affects T-cell function it is conceivable that certain doses favour suppressor T cells whereas others induce helper T cells.

A useful piece of information gathered from these experiments was the dependence of the adjuvant effect of carrageenan on the strain of mouse injected (figures 8,9,10). At a dose of carrageenan (1 mg) which enhanced the response of BALB/c mice, the response of C57BL/6 mice was suppressed. Ishizaka et al. (1978) also reported a genetic dependence for the immunomodulating properties of carrageenan humoral immune responses. Their system differed from the one reported in this thesis in that they assessed the effect of carrageenan treatment on high and low responder mouse strains to a given antigen. Low responders had their immune response boosted by carrageenan treatment whereas high responders were immunosuppressed by carrageenan treatment. In my experiments, BALB/c and C57BL/6 mice produced similar numbers of PFC when injected with SRBC alone.

The response of hybrid mice (figure 10) was different from either parent strain although the shapes

of the hybrid mouse dose-response curves were very similar to the BALB/c curves (figure 11). Hybrid mice reacted with a lower response than C57BL/6 mice and BALB/c mice at low doses of carrageenan, similarly to BALB/c mice at intermediate carrageenan doses, and in between the two parent strains at high carrageenan doses. The murine response to lipopolysaccharide, another immunomodulator, is also under genetic control (McGhee et al., 1979b; Watson et al., 1977). Watson et al. (1977) have shown that a single genetic locus is involved and the response is under additive gene control. It may be that the adjuvant effect of 1 mg kappa-carrageenan is under similar genetic control and therefore this dose produces about half the adjuvant effect in hybrid mice compared to what was found for BALB/c mice. Since many cellular factors, such as regulating genes and gene interactions, can affect the expression of a gene (Lahat et al., 1978), it is difficult to determine the genetic basis for the adjuvant effect of carrageenan. Inbred strains of mice vary in their capacity to produce antibody to a variety of antigens (Coe, 1966; Fink and Quinn, 1953; Ipsen, 1959; Ando and Fachet, 1977; Silver, 1979) and therefore it is not surprising that the capacity to modulate that response also is strain dependent. The ability to

augment delayed hypersensitivity reactions in mice with adjuvant has also been shown to be strain dependent (Snippe et al., 1980). While C57BL/6 and BALB/c mice differ in a variety of traits such as H-2 type, susceptibility to Mycobacteria lepraemurium (Alexander, 1978), and the class of heavy chains produced by their thymocytes (Lahat et al., 1978) it is not possible to clearly identify the essential elements controlling the effect of carrageenan treatment. Studies with the F_1 generation obtained by backcrossing the hybrid mice to each parent and an assessment of the responsiveness of a battery of mouse strains to carrageenan probably would be informative. What is clear from the present studies is that the immunomodulating effects of carrageenan have a genetic basis. Future investigators, therefore, will have to select an appropriate strain depending on what aspect of carrageenan-induced immunomodulation they wish to examine. This genetically determined difference may be responsible for much of the contradictory literature concerning the effect of carrageenan on the immune response.

Timing was another important element determining carrageenan's immunomodulatory effect. The fact that pretreatment with carrageenan suppressed the anti-SRBC response of BALB/c mice (table 10) is in agreement with

results obtained with other immunoenhancing agents. Lipopolysaccharide (Persson, 1977), muramyl dipeptide (Leclerc et al., 1979), Corynebacterium parvum (Ghaffar and Sigel, 1978), polyadenylic acid:polyurydylic acid (Morris and Johnson, 1978), and type II interferon (Sonnenfeld et al., 1978) can all suppress the immune response when administered prior to antigen even though they enhance the response when administered at the same time as antigen. It should be noted that pretreatment of mice with C. parvum can also enhance the anti-SRBC response (Sljivic and Watson, 1977). Ghaffar and Sigel however reported that pretreatment of mice with C. parvum suppressed the anti-SRBC response similarly to the effect of carrageenan in this thesis (C. parvum 24 hr prior to antigen 1% of control response, with antigen 200% of control response, 24 hr after antigen 105% of control response). Possibly treatment with carrageenan prior to antigen favours the induction of suppressor cells, whereas when the polysaccharide is injected with antigen helper cells are induced. Alternatively, carrageenan treatment could cause the polyclonal induction of helper T cells. When pretreated with carrageenan these cells are activated to release short-lived helper factors and then when antigen is injected there is a shortage of these factors. The

majority of the studies reporting suppression of the antibody response due to carrageenan treatment have used animals which were pretreated with the polysaccharide (Ishizaka et al., 1977; Aschheim and Raffel, 1972; Rumjanek et al., 1977) although in one report treatment with 1 mg carrageenan at the same time as antigen suppressed the antibody response (Thomson et al., 1976). In this case, however, both the strain of mice (LACA) and the type of carrageenan they used, an unfractionated kappa-lambda mixture, differed from those used in my experiments, either of which could cause the disparity in results.

The capacity of carrageenan to modulate the humoral immune response of BALB/c mice was also found to be dependent on the type of carrageenan used (figure 12). Very few studies have compared the effect of different types of carrageenan on immune responses. Schorlemmer et al. (1977) found that lambda-carrageenan was more potent than iota-carrageenan in inducing the selective release of macrophage enzymes in vitro. Kappa-carrageenan did not elicit enzyme release. Thomson et al. (1976) reported that lambda- and iota-carrageenan were better suppressors of the immune response than kappa-carrageenan. The data reported in this thesis indicate that kappa- and iota-carrageenan were more potent stimulators of the

immune response than lambda-carrageenan. This is in agreement with the structural similarity of kappa- and iota-carrageenan, and the relative dissimilarity of lambda-carrageenan. The data suggest that the adjuvant effect of carrageenan has a structural basis although it is not clear what that basis is (it may be related to the 3,6-anhydrogalactose unit or the degree of sulfation). At lower doses of carrageenan (10 µg) kappa- and iota-carrageenan differed in terms of their effect of PFC/10^6 spleen cells, suggesting that the mechanism of modulation may either deviate at this dose or have more stringent structural requirements. Experiments using uncharacterized mixtures of carrageenans are difficult to interpret and compare with results using well-defined carrageenans because of this structure-activity relationship.

Dextran sulfate (a sulfated polysaccharide) can act as a polyclonal activator of B cells (Dorries et al., 1974; Persson et al., 1977) and Quan et al. (1978) have shown that iota-carrageenan can act as a B-cell mitogen in vitro. Therefore it seemed possible that carrageenan's adjuvant effect could have been achieved by polyclonal activation of B cells. Fowler and Thomson (1979) noted that rats injected with carrageenan had elevated levels of serum IgM and suggested that carrageenan might be directly mitogenic for B cells. If this were true, the observed

increase in the number of spleen cells might be due to carrageenan-induced proliferation of numerous B cells. The data in this thesis indicate that mice treated with an immunostimulatory dose of kappa-carrageenan alone do not show an increase in background splenic anti-SRBC or anti-HRBC PFC (figure 13). However, when carrageenan was administered with antigen the background response was augmented, although this enhancement was largely due to an increase in the number of spleen cells. Non-specific stimulation of B cells when carrageenan is administered with antigen may occur but the effect is small compared to other B-cell mitogens. Therefore it seems unlikely that carrageenan's mode of action as an adjuvant is due to polyclonal activation of B cells. It is possible that in the presence of carrageenan and antigen very large quantities of helper factors are released causing the expansion of other B cell clones undergoing endogenous antigenic stimulation (as suggested by Bretscher (1978)).

In conclusion, these <u>in vivo</u> experiments have established that carrageenan can act as an adjuvant and that this adjuvant effect depends on:

1. the dose of carrageenan
2. the genetic background of the experimental animals
3. the time of carrageenan administration (relative to antigen)

4. the type of carrageenan

The presence of these factors may explain the conflicting reports found in the literature on carrageenan effects on the immune response. This information together with the studies investigating carrageenan's capacity to activate B cells nonspecifically provide only indirect clues regarding carrageenan's mode of action as an adjuvant. Therefore an *in vitro* method was used to investigate further carrageenan's immunomodulatory activities.

4.2. *In Vitro* Studies

4.2.1 Tube Culture Experiments

The effect of carrageenan on the *in vitro* anti-SRBC response was assessed, since a variety of sulfated polysaccharides, including dextran sulfate, have been reported to enhance *in vitro* anti-SRBC responses (Vogt *et al.*, 1973). In addition there have been no previous reports showing the enhancement of *in vitro* antibody responses by carrageenan and it was of interest to assess this activity. Kappa-carrageenan, added to cultures of BALB/c spleen cells, enhanced the anti-SRBC response (figure 14). This enhancement was optimal at a dose of 5.0 $\mu g\ ml^{-1}$ whereas the polysaccharides in Vogt *et al.*'s

(1973) study augmented the PFC response in the dose range of 100-500 μg ml^{-1}. Carrageenan (0.05-50 μg ml^{-1}) increased the number of cells per culture and although the increase was small it was statistically significant at several dose levels. A high dose of carrageenan (500 μg ml^{-1}) was cytotoxic for BALB/c spleen cells *in vitro*.

The adjuvant effect of carrageenan *in vitro*, like the effect *in vivo*, was dependent on the genetic background of the experimental animals (figures 14, 15, and 16). The pattern of responsiveness by C57BL/6 spleen cells, compared to BALB/c spleen cells, was similar to the *in vivo* situation in that higher doses of carrageenan which enhanced the anti-SRBC response of BALB/c spleen cells did not significantly augment the response of C57BL/6 spleen cells. However, the response of hybrid mouse spleen cells to carrageenan more closely resembled the C57BL/6 response then the BALB/c response (figure 17) which was in disagreement with the *in vivo* data. A high dose of carrageenan (500 μg ml^{-1}) was also cytotoxic for C57BL/6 and hybrid mice. Since carrageenan can precipitate protein *in vitro* (Anderson, 1967) it is possible that this cytotoxicity may be due to a lack of soluble protein in the tissue culture medium causing the cells to starve. These studies indicate that carrageenan

can modulate the *in vitro* anti-SRBC response of BALB/c, C57BL/6, and hybrid mice. The pattern of this modulation, particularly in the hybrid mice, was dissimilar to that observed *in vivo* suggesting that direct addition of carrageenan does not provide a good model for the *in vivo* immunomodulating effects of carrageenan. These data suggest that the genetic differences observed *in vivo* are dependent, in part, on phenomena which cannot be duplicated *in vitro* such as lymphocyte recruitment or induction of hormone release.

Different types of carrageenan vary in their capacity to modulate the *in vitro* immune response (figure 18). The data indicate that neither iota- nor lambda-carrageenan enhanced the anti-SRBC response of BALB/c spleen cells at a dose at which kappa-carrageenan stimulated the response. The fact that iota-carrageenan did not enhance the *in vitro* immune response, which was at variance with the *in vivo* results, suggested that either a different mechanism of modulation by carrageenan occurs *in vitro* or a more stringent structural requirement is necessary *in vitro*.

Whatever the mechanism of carrageenan's modulation of the *in vitro* immune response, it was not due to the polyclonal activation of B cells. Enhancing doses of kappa-carrageenan did not elevate the background anti-red

blood cell response of BALB/c mice when administered alone or with antigen (figure 19). The lack of background stimulation when carrageenan was added with antigen *in vitro*, in contrast to what was found *in vivo*, may be due to the fact that the stimulatory dose of carrageenan *in vitro* did not significantly increase the number of cells per culture.

In summary, these data show that carrageenan can act as an adjuvant *in vitro*, but it does not appear that the tube culture system provides a good model for carrageenan's *in vivo* mode of action since many of the results obtained *in vitro* differ from those *in vivo*.

4.2.2 Marbrook Experiments

An important difference between the tube culture experiments and the *in vivo* experiments was the environment under which carrageenan interacted with the immune system. *In vivo*, numerous environmental factors exists which do not occur *in vitro*. In an effort to minimize these differences, mice were treated with carrageenan *in vivo* and the capacity of spleen cells from these mice to modulate the immune response was assessed *in vitro*.

Spleen cells from mice which had been treated with 1 mg kappa-carrageenan *in vivo* released a soluble

enhancing factor(s) which augmented the in vitro anti-SRBC response (figure 20). The amount of enhancement was dependent on the number of carrageenan-treated cells present but the increase in the top chamber response was not linearly related to the number of carrageenan-treated cells present. This suggested that more than one cell population was involved in the production of such a factor(s). It is conceivable that a second population of cells (population #2) facilitated the release of the soluble factor(s) by population #1. Population #2 may be present in limiting amounts and the full effect of these cells only becomes apparent at high numbers of cells (10^7) thus causing a steep increase in the number of PFC developing in the top chamber. Alternatively, the production of the soluble factor(s) may depend on cell density.

The probability that the enhancement of the top chamber anti-SRBC response was mediated by a soluble factor(s) is supported by the fact that the two chambers were separated by a dialysis membrane and also that supernatants produced by carrageenan-treated cells enhanced the top chamber response (figure 23). The fact that supernatants could enhance the top chamber response suggests that the production of this factor(s) was not dependent on "signals" from the antigen-stimulated cells

in the top chamber. The enhancement produced by the soluble factor(s) is not likely to be due to the screening out of suppressive factors by the dialysis membrane since preliminary observations indicated that carrageenan-induced supernatants could enhance the anti-SRBC response when added directly to tube cultures of normal spleen cells and SRBC. Besides, many suppressive factors have a low molecular weight (Watson, 1973; Calderon et al., 1974) and therefore should be able to cross the dialysis membrane.

It should be noted that the soluble factor(s) did not augment the response in the top chamber in the absence of antigen. This suggests that the carrageenan-induced soluble factor(s) may act only on antigen triggered cells, analagous to the second signal in the two-signal model of the antibody response.

The possibility that some carrageenan was transferred with the spleen cells that were placed in the bottom chamber cannot be excluded. While such transferred carrageenan might stimulate the cells in the bottom chamber, it is not likely that this carrageenan could alter the response of the top chamber cells directly. Native carrageenan is a large molecule (molecular weight of at least 100,000 daltons) and therefore could not cross the dialysis membrane. In addition, since mammalian cells cannot degrade carrageenans, it is

unlikely that small fragments of the polysaccharide that could cross the dialysis membrane would be produced.

The similarity of the *in vivo* effect of carrageenan and the *in vitro* effect in the Marbrook cultures suggests strongly that *in vivo* enhancement is attributable to release of a soluble factor(s). The dose of carrageenan which suppresses the *in vivo* immune response (10 µg) does not have a similar effect *in vitro* (figure 21), although, the overall shapes and magnitude of the *in vivo* and *in vitro* dose-response curves are similar (figure 22). Note that low doses of carrageenan (0.1 µg) which modestly stimulate the *in vivo* anti-SRBC response also boost the *in vitro* anti-SRBC response. These results suggest that the adjuvant effect of carrageenan *in vivo* is related to the release of soluble enhancing factor(s) (assessed *in vitro*). The evidence for this hypothesis is indirect and would have to be tested directly (e.g. see if an antiserum to soluble factor(s) can abrogate the *in vivo* adjuvant effect of carrageenan). If such direct evidence supports the hypothesis, then the Marbrook vessel model of carrageenan's adjuvant effect is a valid one.

Carrageenan-treated cells enhanced the anti-SRBC response of the top chamber cells when suboptimal, optimal, and supraoptimal amounts of SRBC were added to

the top chamber. The magnitude of the response in the top chamber depended on the dose of SRBC which is in agreement with typical antigen dose-response curves. The amount of enhancement (as a percentage of the control response) induced by the carrageenan-treated cells was dependent on the amount of antigen in the top chamber. Carrageenan-treated cells caused a maximal enhancement when the response in the top chamber was at a minimum ($5x10^7$ SRBC) and enhancement was at a minimum when the response in the top chamber was at a maximum ($5x10^6$ SRBC). This suggests that under suboptimal conditions the top chamber cells have a greater requirement for the carrageenan-induced soluble enhancing factor(s).

Figure 24 also indicates that the production of the soluble factor(s) does not require an anti-SRBC response in the bottom chamber hence the production of enhancing factor(s) is not dependent on some "signal" from the top chamber (either from the antigen or the spleen cells) which is interpreted differently by carrageenan-treated as opposed to normal cells. The production of the soluble factor(s) is dependent on *in vivo* treatment with the appropriate dose of carrageenan.

That the effect of the soluble factor(s) was dependent on the magnitude of the top chamber response was further supported by the data presented in table 12.

During the course of the Marbrook vessel experiments, occasionally carrageenan-treated cells did not enhance the response in the top chamber. When the data was selected and examined it was found that carrageenan-treated cells could not elevate the response in the top chamber when the response in that chamber was high. When the top chamber anti-SRBC response was low carrageenan-treated cells clearly augmented the response. When all of these data were pooled carrageenan-treated cells were found to induce statistically significant enhancement of the top chamber response although the amount of enhancement was less than that obtained when low responding cultures alone were compared. It can also be seen that a significant amount of this enhancement was due to an increase in the number of cells in the top chamber when carrageenan-treated cells were present in the bottom chamber. This suggests that the factor(s) may act by expanding a population of spleen cells in the top chamber, or by slowing the death rate in that chamber.

In a closed system such as a Marbrook vessel, it is apparent that there must be a limit to the immune response. This limit is imposed by the number of spleen cells and nutritional factors. The limit in my culture appears to be in the vicinity of 2,500 PFC/culture.

Carrageenan-treated cells enhanced the response of low response cultures to this limit. This suggests that the carrageenan-induced soluble enhancing factor(s) is, or simulates, a normal physiological product which is in short supply in low but not high response cultures. By reducing the response of high response cultures, by reducing the number of top chamber cells, it can be shown that the carrageenan-treated cells augmented the response (figure 25). The carrageenan-induced factor(s) then became limiting (in the top chamber) and therefore the bottom chamber cells could augment the response. The enhancement was dose-dependent.

4.2.3 The Nature of the Soluble Enhancing Factor(s)

The data in table 13 suggested that the soluble enhancing factor(s) was produced by a non-adherent thy 1^+ cell. Treatment of carrageenan-treated spleen cells with anti-thy 1.2 and complement abrogated the enhancing effect of carrageenan-treated cells (treatment with complement alone had no effect). This treatment should kill thy 1.2^+ cells, without lysing B cells and macrophages. T-cell depleted spleen cells like non-adherent spleen cells gave much reduced PFC response to SRBC. When carrageenan-treated spleen cells were treated with carbonyl iron,

enhancement of the top chamber response still occurred. Carbonyl iron treatment principally removes adherent and phagocytic cells such as macrophages but can also significantly deplete B cells (Rumpold et al., 1979; Fernandez and MacSween, 1977). Thus, the soluble factor(s) producing population consists mainly of non-adherent T cells. Collectively then, these data suggest that a non-adherent T cell produces the soluble enhancing factor(s). It is possible that a population of macrophages produce the factor(s) upon interaction with T cells although since carbonyl iron treatment is effective at removing macrophages and since the removal of adherent cells with the magnet was repeated I view this as unlikely.

Carrageenan-treated T cells mixed with normal adherent cells were still capable of enhancing the anti-SRBC response in the top chamber (table 14). This is in agreement with the data shown in table 13 and shows that normal adherent cells, after exposure to anti-thy 1.2 and complement, do not inhibit production of the soluble enhancing factor(s). Table 14 also shows that T-cell depleted, carrageenan-treated, spleen cells combined with normal non-adherent spleen cells could enhance the top chamber PFC response. By themselves each of these populations was incapable of augmenting the top chamber

response. From this information, one can devise a model to explain the mechanism of action of carrageenan's adjuvant activity.

1. In vivo carrageenan initially interacts with the macrophage either by binding to its surface or by being engulfed by these phagocytic cells.
2. As a consequence of this carrageenan-macrophage interaction the macrophage is, in some way, activated. This activated macrophage is capable of activating T cells.
3. The activated T cells release a soluble enhancing factor which can cross the dialysis membrane and enhance the PFC response (a similar event occurs in vivo).
4. The macrophage-T cell interaction must involve either cell contact or the release of a high molecular weight soluble factor which cannot cross the dialysis membrane and therefore carrageenan-treated macrophages cannot augment the top chamber response.

In the following section I will discuss the above four.

1. Carrageenan interacts with the macrophage

It would not be surprising if the carrageenan initially interacted with macrophages since carbohydrates are known to bind nonspecifically to macrophages (Weir and Ogmundsdottir, 1979). In addition, carrageenan has been shown to be engulfed by macrophages *in vivo* (Catanzaro *et al.*, 1971) and *in vitro* (Allison *et al.*, 1966). This carrageenan-macrophage interaction event may be under genetic control. Lukic *et al.* (1975) have shown that BALB/c macrophages are more efficient at processing certain antigens that DBA/2 macrophages. If BALB/c macrophages differ functionally from C57BL/6 macrophages, then the finding that high doses of carrageenan have a different effect in these two strains may be related to genetically determined macrophage activity.

2. Macrophages are activated

Several other adjuvants have been shown to enhance the immune response by affecting macrophage function (Unanue *et al.*, 1969; Sljivic and Watson, 1977; McGhee *et al.*, 1979a; Fevrier *et al.*, 1978). In most cases however, it is unclear how this alteration of macrophage activity modulates the immune response. Macrophages are

known to produce factors which enhance the in vitro antibody response by acting on T cells (Young and McIvor, 1980; Erb et al., 1976; Calderon et al., 1975) hence the kind of model I have described is not without precedent.

3. Activated T cells release a soluble enhancing factor

T cells are known to produce low molecular weight soluble enhancing factors (<20,000 daltons) under the influence of allogeneic (Feldman and Basten, 1972), antigenic (Rubin and Coons, 1972; Feldmann, 1972), and immunomodulatory compounds (Sanford et al., 1979). Furthermore, the release of these factors may be caused by soluble macrophage-derived factors. Macrophages release the lymphokine interleukin 1 which in turn causes T cells to release interleukin 2 (Paetku et al., 1980). Interleukin 2 is capable of augmenting the development of antigen-triggered helper T cells involved in antibody responses (Paetku et al., 1980). Other adjuvants, such as lipopolysaccharide (Smith et al., 1980) and muramyl dipeptide (Oppenheim et al., 1980), can induce production of interleukin 1 and Stamenkovic et al. (1979) have suggested that carrageenan may also be able to induce this factor. While interleukin 2 has been reported to have a molecular weight of about 40,000 daltons (Paetku et al.,

1980), Altin and DiSabato (1980) have recently shown that interleukin 2 occurs as a heterogenous group of molecules with the low molecular weight forms weighing 4,000-4,700 daltons. A molecule of this size could certainly cross a dialysis membrane.

4. The macrophage factor cannot cross the dialysis membrane

Interleukin 1 occurs in two molecular forms, one having a molecular weight of 16,000-22,000 daltons, and the other weighing 60,000-70,000 daltons (Oppenheim et al., 1980). These molecules would be too large to cross the dialysis membrane and thus the interleukin model fits the available data. Preliminary data that I have obtained indicate that the carrageenan-induced enhancing factor(s) cannot enhance the anti-SRBC response of spleen cells which have been depleted of T cells. This further suggests that the soluble enhancing factor(s) is interleukin 2 since this lymphokine acts on T cells to affect B-cell activity (Harwell et al., 1980). There are many reports concerning soluble factors, produced by T cells, which enhance the antibody response of T-cell depleted spleen cells (Watson, 1973; Bernabe et al., 1979; Askonas et al., 1974; Taussig and Munro, 1976; Watson et al., 1979a; Hunter and Kettman, 1974) and if my

preliminary data are correct these can be ruled out as being produced by the carrageenan-treated cells.

It is also possible that macrophages present carrageenan to T cells which causes the T cells to release soluble enhancing factor(s). This event could occur *in vitro* in the mixing experiments. However in view of the above argument I favour the view that carrageenan induces the production of interleukin 1. If this is true the cellular target for carrageenan's adjuvant activity is the macrophage.

Interferons are known to be induced by high molecular weight, highly charged, molecules (Finter, 1973). These immunomodulating molecules are induced by lambda- but not by kappa-carrageenan (Turner and Sonnenfeld, 1979). Since mouse interferons, although heterogenous in molecular weight, are larger than 20,000 daltons (Finter, 1973) it is unlikely that they would be able to cross the dialysis membrane even if kappa-carrageenan induced these molecules.

Further work would have to be done in order to test my hypothesis that carrageenan enhances the immune response by inducing interleukin 1. Supernatants, produced by carrageenan-treated macrophages, could be physiochemically characterized and compared to standard interleukin 1 preparations. The effect of these

supernatants on T cell production of soluble factors could be similarly examined. The injection of carrageenan-induced, purified soluble factors into mice could be compared with injecting carrageenan into mice with respect to the immune response.

The mechanism of carrageenan's adjuvant effect, if it is as I have described it, is complex. It appears to act on one cell which in turn effects a second cell which then leads to production of a soluble factor that modulates the activity of a third cell. This complexity may explain why the mode of action of adjuvants in general has been so difficult to explain. Because of recent advances concerning the activity of soluble regulatory molecules it may now be possible to explain more clearly the mode of action of a variety of adjuvants.

SUMMARY

1. Carrageenan has been shown to enhance the *in vivo* primary humoral immune response to SRBC. This enhancement was dependent on the dose of carrageenan administered, the type of carrageenan given, the genetic background of the experimental animals, and the time of carrageenan administration relative to antigen.
2. Carrageenan has also been shown to enhance the *in vitro* primary humoral immune response to SRBC when directly added to spleen cell cultures. This enhancement was dependent on the dose of carrageenan, the type of carrageenan and the genetic background of the spleen cell donors.
3. Kappa-carrageenan, at immunostimulatory doses, did not act as a polyclonal activator of B cells *in vivo* or *in vitro*.
4. Spleen cells from mice treated with immunostimulatory doses of carrageenan *in vivo* released a soluble factor(s) which enhanced the *in vitro* anti-SRBC response. Since the release of these enhancing factor(s) *in vitro* was correlated with the dose of carrageenan administered *in vivo* it was suggested that

carrageenan-mediated enhancement of the *in vivo* immune response was due to release of soluble enhancing factor(s).

5. Since soluble enhancing factor(s) was produced by a nonadherent thy 1^+ cell and cell-cell interactions were involved in its production, it was suggested that the carrageenan induced soluble factor may be the lymphokine, interleukin 2.

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