A DESIGN TO STUDY THE INCIDENCE, CELL-MEDIATED IMMUNITY AND MORBIDITY ATTRIBUTABLE TO CYTOMEGALOVIRUS AMONG RENAL TRANSPLANT AND DIALYSIS PATIENTS

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Abstract

Cytomegalovirus (CMV), one of the herpes viruses, is a common infectious agent among immunosuppressed renal transplant patients. Although CMV infection is usually mild or asymptomatic among general population, it can result in critical illness, allograft rejection and even death among transplant patients. This thesis designs experiments for the study of CMV with respect to its incidence, morbidity and mortality as well as its effects on the cellular immunity among renal transplant and hemodialysis patients. The principles of incidence study are outlined, the concept of sensitivity, specificity and predictive value of a test explained, and the difference between the laboratory and epidemiologic usage of these terminologies are discussed. The sources of error in Lymphocyte Stimulation Test as an instrument to assess cell-mediated immunity are idscussed. The complexity of morbidity study is also discussed. Sample size determinations for the incidence study, morbidity as well as cellular immunity studies are discussed. Statistical analysis of the data (when available) is discussed.

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CHAPTER ONE

INTRODUCTION

1.1 Introductory Explanation

Prior to 1950, there was very little that could be done for patients with end-stage renal disease. With the advent of hemodialysis and renal transplant in the 1950's, the outlook for these patients became more promising.[1-3] The addition of immunosuppressive agents to the management of transplant patients reduced rejection episodes significiantly.[1-5]

However, because of immunosuppression, and possibly in association with chronic renal disease,[2-5] infection became a major factor in the morbidity and mortality experienced by thses patients.[1-3,5,41] Although their humoral immunity may be normal quantitatively, the cell-mediated immunity is often depressed. These patients are, therefore, prone to viral infections particularly the herpes virus group. The viral infection can then predispose to bacterial infection to which these patients may succumb.

Cytomegalovirus (CMV) is the commonest virus incriminated as an infectious agent among these patients. Although the mortality attributable to this virus among renal transplant and dialysis patients is small, the morbidity can be considerable. Pneumonia, febrile illness, hepatitis and even rejection episodes have been associated

with cytomegalovirus infections. It is true, however, that most of these renal transplant and dialysis patients who are exposed to and who develop antibodies to CMV are asymptomatic.

There are various reports in the literature about the prevalence of CMV antibodies among these patients. Some of these reports use "incidence" interchangeably with "prevalence" of CMV. The few that attempted the study of incidence of CMV among renal transplant and dialysis patients either have too small a sample size or do not exclude the presence of CMV in a silent form among their study sample even before the inception of their study.[11-13]

In addition, repeated studies of morbidity and mortality sometimes have the problem of not knowing the initial state of these patients with respect to prior CMV infection. Even when these are known, comparing the results obtained from studying reactivated infections with that of primary infections may not be valid.

These shortcomings are compounded by the fact that, in some instances, many variables (age, sex, socioeconomic status, source of kidney, duration of dialysis and extent of blood transfusion pre-transplant) which appear to be important in determining acquisition of CMV post-transplant are not taken into account in the analysis of the data.[10,12,13]

The source of CMV post-transplant remains an

important debate in the literature. Suspected sources include donated kidneys, blood transfusions, dialysis center and re-activation of latent CMV in an immunosuppressed individual. The clinical implications of the source of CMV during post-transplant renal patients is discussed in section 3.3.

1.2 Aims of the Thesis

This thesis proposes a method for studying the CMV among renal transplant and dialysis patients. This design is that of a cohort analytic study. Methodologic issues such as initial state of the study population (sample), sample size and co-morbidity are clearly defined and discussed.

Specifically the thesis is designed to study, among renal transplant and dialysis patients:

(a) the incidence of CMV,

(b) the natural history of CMV infection (morbidity and mortality), and

(c) the effects of CMV infection on cell-mediated immunity.

1.3 Format of the Thesis

Chapter Two: describes the general consideration of the impact of transplantation and dialysis on the outcome of end stage renal disease (defined in section 2.1) today, the immunology of transplantation and the need for

immunosuppression among these patients. This chapter also deals with the role of infection in the morbidity and mortality among these patients; and the nature of the infectious agents.

Chapter Three: deals with the role of cytomegalovirus in end-stage renal disease, its virology and immunology as well as a critical review of the literature on the subject of cytomegalovirus in renal transplantation and dialysis.

Chapter Four: describes the overall experimental design to study incidence, morbidity and cell-mediated immunity among these patients. The chapter deals with the sample size determination, describes the study population and discusses the advantages and disadvantages of using a single programme as a source of the study population. Criteria for admission to the study is set out in this chapter. Administrative strategies for management of the study is discussed in this chapter.

Chapter Five: deals with the incidence study. The essential terminologies relevant to the study of incidence of CMV are defined in this chapter. The principles involved in the study of incidence such as the instrument for measuring the outcome of interest are discussed. The problem of what to do with those who drop out of the study or those who are entered in the middle of the study as well as those who may die while

the study is still in progress is discussed here. The experimental design to study incidence, the principles of immune adherence hemagglutination (IAHA) tests are dicussed. Dummy tables for data record are presented here.

Chapter Six: is devoted to the study of cell-mediated immunity among these patients. The principles of lymphocyte stimulation test and radioactive counting are discussed here. The chapter also discusses the problems involved in these tests. Dummy tables for data record are presented.

Chapter Seven: pertains to the study of morbidity and mortality among renal transplant and dialysis patients attributable to CMV infection. In this chapter, morbidity, mortality are defined, and morbid factors are discussed. Co-morbid factors are also considered. The experimental design for the study of morbidity attributable to CMV is discussed; dummy tables for data record are presented.

Chapter Eight: Here, statistical principles for analysis of data pertaining to incidence study, morbidity and mortality data as well as for data on radioactive counts are discussed. Dummy tables and corresponding statistical analyses are discussed.

Chapter Nine: pertains to the budget for the study and justification for such a budget. The chapter also deals with ethical issues involved in the study.

Chapter Ten: This portion discusses the conclusions arrived at by the thesis.

Chapter Eleven: Appendix: Details of statistical explanation regarding log-linear models is discussed. An example of informed consent is presented. Then references are listed.

CHAPTER TWO

2.1 General Consideration of the Impact of Renal Transplant and Hemodialysis on the Outcome of End-Stage Renal Disease

End-stage renal disease is characterised by an inability to maintain normal biochemical architecture of the body fluid under normal living conditions. It results in death unless there is an extra-renal means of maintaining the body's fluid biochemical balance. In the past, the patient's with end-stage renal disease died, usually of hyperkalemia (high serum potassium) and/or pulmonary edema. Anemia, heart failure, pericarditis, hemorrhage, and an infection were other causes of death.

With careful blood transfusions, and dietary manipulations, the outlook for these patients improved a little. In selected patients without progressive or advanced vascular damage, the above modalities of treatment may lead to about seven years of survival only. However, these management measures were still conservative. The overall outlook for these patients was still grave.

As a result of advances in immunology, nephrology, and surgical urology, renal transplantation in the 1950's and hemodialysis in the 1960's revolutionalised the outlook for these patients. The 1973 international transplant registry figures showed that at 3 years, 71% of sibling

renal graft and 59% of parental were still surviving. Current practice emphasizes early diagnosis of rejection; treatment and allograft nephrectomy is performed if rejection is not immediately reversible. The patient can then depend on dialysis for normal biochemical homeostasis. Handled in this fashion, patient survival rather than kidney survival is the priority.

An example of the impact of hemodialysis on end-stage renal disease was presented by Jean Hamburger et al. (1968).[1] The data showing the impressive reduction in per cent mortality among anuric (postpartum) women who were dialyzed as compared to those who were not (see Table 2.1 below).

TABLE 2.1

Influence of the Artificial Kidney on the Mortality of Severe Postpartum Anuria

(12 days + BUN 400 mg%)

Period	Number of patients	Cured	Died	% Mortality
1952-1953	62 patients before artificial kidney	16	46	74%
1955-1956	62 patients treated by artificial kidney	57	5	8%

From: "Nephrology" by Jean Hamburger et al.[1]

The world statistics of kidney transplantation (1959-1969) also confirms the general improving trends whether the donor is a sibling, parent or cadaver. See Tables 2.2 below.

TABLE 2.2

% of Graft Survival at 2 Years

Calculated by Acturial Method[1]

Donor	March 1959 to March 1965	January 1966 to January 1968	January 1967 to January 1969
Sibling	52%	76%	81%
Parent	32%	57%	73%
Cadaver	14%	38%	40%

From: "Renal Transplantation: Theory and Practice" by Jean Hamburger et al. (1972)[1]

The data from the Canadian Renal Failure Registry (1976)[82] indicates that the probability of surviving 12 months on either home or hospital is better than 95% (Figure 2.1, page 11). For transplant patients, a distinction must be made between patient survival and graft survival. As indicated above, the practice today is to keep the patients alive and lose rejected allografts, if rejection is not easily reversed. Therefore, the patient survival is improving more markedly than allograft survival. However, with better immunologic workup, the allograft survival has also improved

because rejection episodes that would result in nephrectomy being performed is occuring less frequently. The data from the Canadian Renal Failure Registry (1976) for renal transplant patients is present in Figures 2.1, 2.2, 2.3, and 2.4 (pages 11 - 14). From these figures, it is noted that the 12 months patient survival for cadaver transplants is about 90%. Although one year is a relatively short time, this figure is still better than 40% two year survival reported in 1967-69 in the world statistics of kidney transplantation. On the other hand, the probability of allograft surviving 12 months is only about 60%. As one would suspect, Figure 2.3 indicates that the older the patient, the less the probability of surviving 12 months when transplanted with a cadaver kidney. The primary diagnosis in a renal failure individual may make a difference to the outcome of cadaver renal transplant. Figure 2.4 illustrates this point. It appears that glomerulo-nephritis gives the best chance of patient survival when the patient receives a cadaver transplant.

A more recent report[7] indicates a 4% mortality among renal transplant patients. This mortality rate among renal transplants is getting lower because renal transplant and dialysis continues to improve the outlook for these patients.

Although the morbidity/mortality rates were higher among renal transplant patients than the dialysis patients,[2,6] renal transplantation may be a better method of treating

FIGURE 2.1

Patient Survival on Initial

Mode of Dialysis in 1976

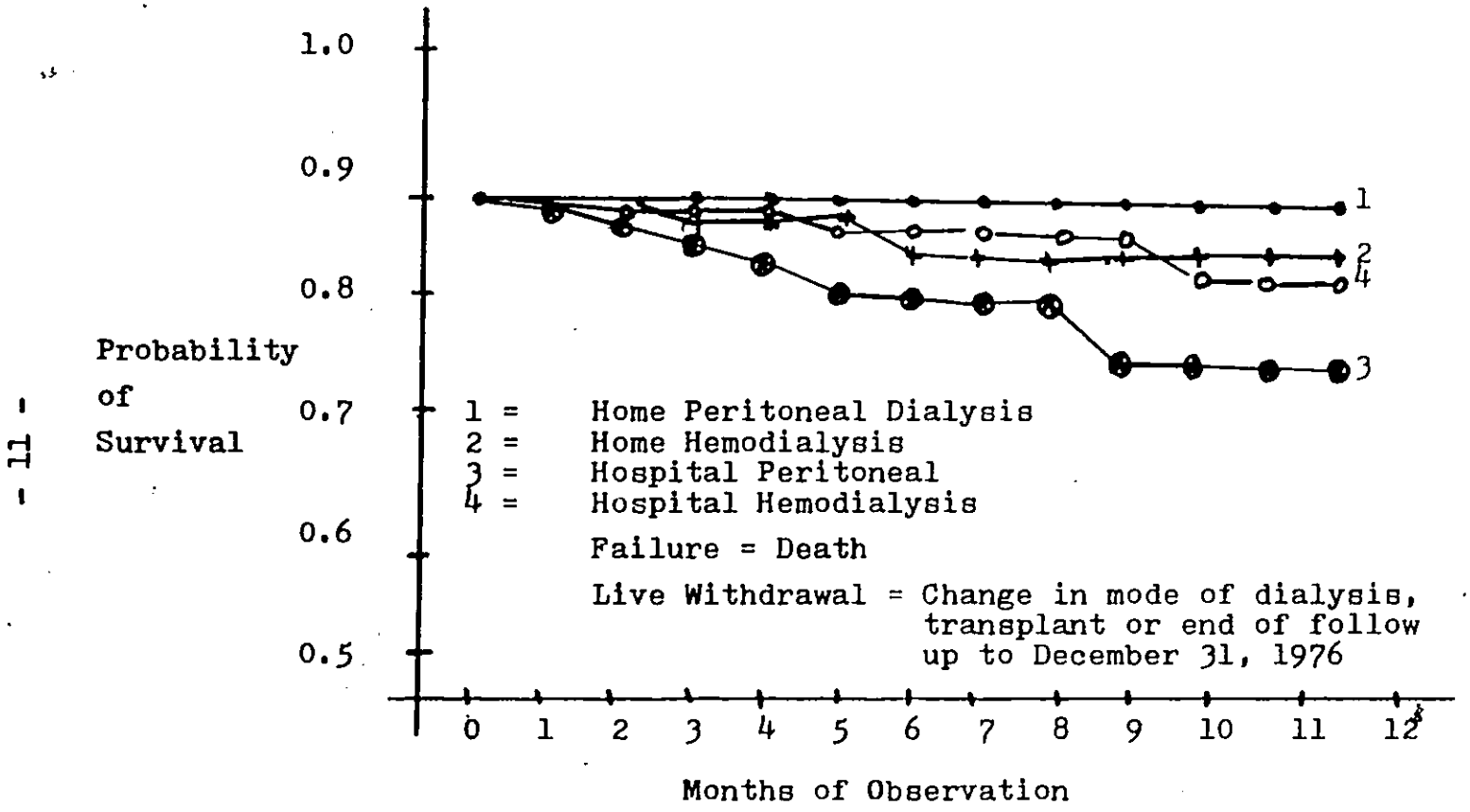


FIGURE 2.2

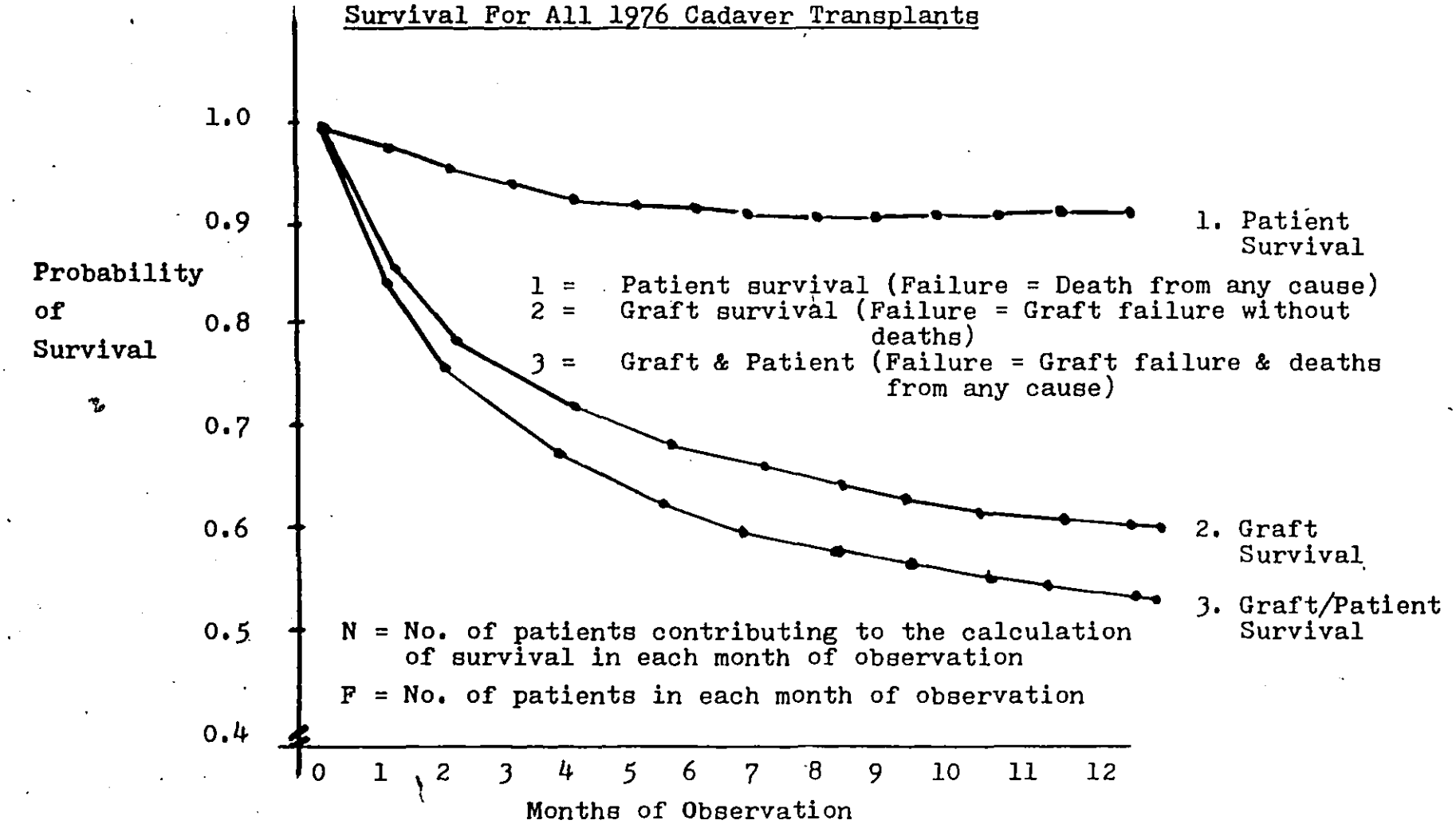

Survival For All 1976 Cadaver Transplants
Probability of Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
1 = Patient survival (Failure = Death from any cause)
2 = Graft survival (Failure = Graft failure without deaths)
3 = Graft & Patient (Failure = Graft failure & deaths from any cause)
1. Patient Survival
2. Graft Survival
3. Graft/Patient Survival
N = No. of patients contributing to the calculation of survival in each month of observation
F = No. of patients in each month of observation
0 1 2 3 4 5 6 7 8 9 10 11 12
Months of Observation

FIGURE 2.3

Patient Survival For 1976 Cadaver Transplants By Age

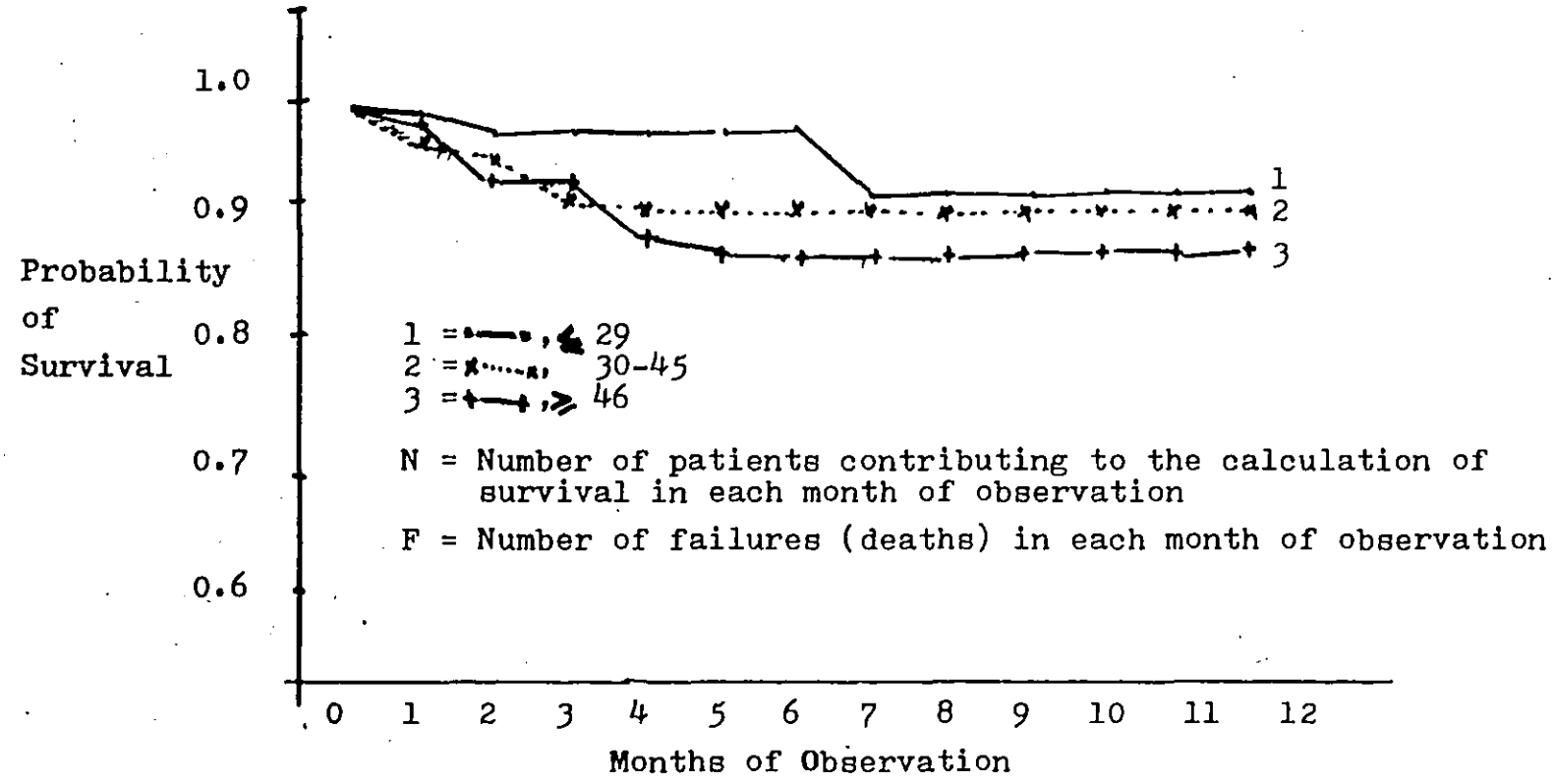
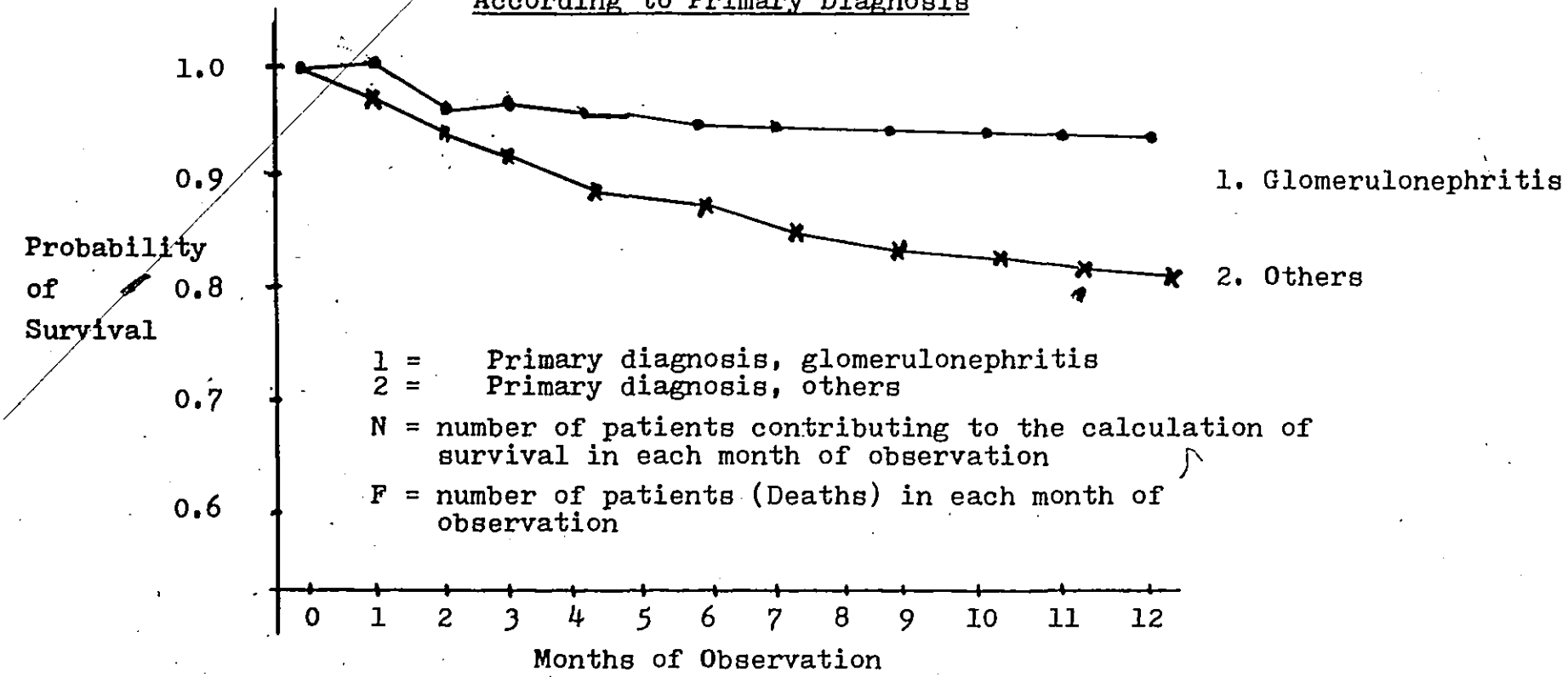


FIGURE 2.4

Patient Survival For 1976 Cadaver Transplants

According to Primary Diagnosis



end-stage renal disease because successful transplantation is a permanent reconstitution. The goal, therefore, is to maintain these patients on dialysis until suitable kidneys are available. Also, even when transplanted, dialysis remains the alternative mode of treatment in cases of allograft rejection.

2.2 Transplant Immunology

Renal transplant is an ideal way of restoring a normally functioning kidney to an individual who has end-stage renal disease. This goal is not always achieved since the transplanted kidney may be rejected by the recipient. This is usually the case when tissues are transplanted between two genetically unrelated individuals. The recipient regards the transplanted tissue (kidney) as "foreign" and mounts a host versus graft reaction, destroying the grafted tissues. This type of reaction is a serious problem in renal transplantation and is one of the major causes of morbidity among renal transplant patients. The closer the donor is to the recipient immunogenetically, the less the tendency to reject. Identical tissue would be ideal.

There are three types of allograft rejection:

2.2 (a) Hyperacute Rejection

This is an immediate type of reaction. The host and the graft usually have major differences genetically. As soon as there is vascular link between the donor and the

recipient immediate reaction occurs. The kidney turns purple and is congested. The reaction appears to be complement mediated immune complex (Arthus) type of reaction. This type of reaction may occur in ABO (blood group) incompatibility or if the recipient or the donor has been sensitized by blood transfusions previously. With good tissue typing, this sort of reaction should occur rarely.

2.2 (b) Acute Rejection

This type of reaction occurs within 5 - 50 days post-transplant. This is the most common type of rejection. It occurs in approximately 50% of all renal transplantations.[1] This is early rejection in which the kidney that has started functioning normally post-transplant then deteriorates progressively. If there is no anti-rejection intervention, renal failure will occur.

2.2 (c) Chronic Rejection

This is a late onset, insidious type of rejection that occurs more than three months post-transplant. It may not become clinically apparent until about six months post-transplant.[1] However, the end result of this type of rejection is also renal failure.

2.3 Immunosuppression in Renal Transplant Patients

Allograft rejection is the most common non-surgical cause of morbidity post-transplant.[1,9] Since rejection is

an immunologic process,[1,8,9] an attempt to suppress this immunologic response is logical. This suppression will minimize the host's attempt to reject the graft.

Different immunosuppressive maneuvers which have been tried include irradiation,[4,5] anti-lymphocyte serum,[1,2] or globulin fraction,[1,2] chemical inhibitors (drugs such as azathioprine) of immune response,[1,2,5,8,12,15,19] and physical removal of lymphocytes by lymphocyte drainage through the thoracic duct.[7] These measures achieve varying degrees of immunosuppression. Currently, immunosuppressive drugs are preferred to other measures cited above. Azathioprine and corticosteroids are standard drugs used to reverse rejection episodes.[1,2,8]

On the one hand immunosuppression minimizes the tendency to rejection, but on the other hand the host loses its ability to fight infection, which then becomes a major problem. Infection is next to rejection as a cause of morbidity among renal transplant patients.[1,2] Not only bacteria but also viral infections are common. The problem of infection is often compounded by the fact that its clinical presentation among these immunosuppressed patients may be atypical.

In the series reported by Rifkind,[41] infection caused 27 out of 33 deaths among the patients. In the same series, infection of varying degrees of severity complicated the post-operative course of virtually all the patients in

that study group. The only two who had no infections at all were identical twins who did not receive immunosuppressive therapy.

With suppression of cell-mediated immunity, viral infections and infection by opportunistic organisms such as candida and pneumocystis carinii become very important. Viral infections may cause death, or predispose the patients to bacterial infections.

The infectious agents frequently reported include: staphylococcus, pseudomones species and enteric gram negative bacilli.[9,41] Thirty-five out of fifty-two infections reported by Rifkind[41] occurred post-transplant. Thirty of the thirty-five infections occured in patients with increase in immunosuppressive therapy in an attempt to reverse apparent allograft rejection.

Many of these patients had viral infections, and then were secondarily infected by bacteria. Chest infection was very common among them. The commonest viral agents isolated among these patients were herpes viruses particularly cytomegalovirus.[40]

CHAPTER THREE

CYTOMEGALOVIRUS AMONG RENAL TRANSPLANT AND DIALYSIS PATIENTS

3.1 Virology of Cytomegalovirus

Cytomegalovirus (CMV) belongs to the herpes virus family. Its particles are indistinguishable from those of herpes simplex virus, varicellar-zoster virus, or that of other members of the herpes virus family.

CMV is labile to low pH, fat solvents and temperature. Rapid freezing techniques will preserve the virus (-60^{o} - $-80^{o}C$). At $-20^{o}C$ the virus is inactivated. It can be isolated by direct innoculation of fresh materials into cultures of human fibroblasts.

Cytomegalovirus is very common among the general population. Various studies showed a prevalence of 35-50% among the normal adult population of antibody detection.[21-26] There are few reports that show up to 65% of general adult population as having CMV antibodies.[22,43] Various factors determine the prevalence of this virus in the general population. Age is important. There is almost a linear relationship between age and the prevalence of CMV antibody. However, by the age of 40-50 years, there is little further increase in the number of persons contacting this virus.[44]

Virus excretion is higher among females especially of childbearing age than males[31] and it is also higher among people of low socioeconomic groups than the middle class - upper class.[92] It is worldwide in distribution.

CMV antibody is present for life though the level may fluctuate. Though very common among the general population,[20-27] the virus is almost always subclinical.[10] Antibody detection by serology is a satisfactory method of making a diagnosis of past infection. Culture of urine or throat swabs may not be positive in patients who have had previous exposure.

Among the immunosuppressed individuals, reactivation of latent CMV infection is more frequent than that experienced by the general population. They present with clinical manifestations of CMV infection more often than the general population (for example the renal transplant patients on immunosuppressive agents).

Those who require repeated blood transfusions have higher rates of antibody acquisition than the general population.[29]

3.2 Nature of CMV Infection

CMV, like other herpes viruses, can cause primary infection. Latency and reactivation of the latent form can occur in CMV infection. All these features are not unusual for other herpes viruses. Various theories have been

presented to explain the persistency of CMV infection. Lang et al.[59] showed that in the presence of CMV antibody following congenitally acquired CMV, the virus may persist for years. There may be persistence of CMV following clinical infections among adults as well. Failure of T-cells (defined in Chapter Seven) to recognize and eradicate the infected cells has been suggested as the mechanism for the CMV persistence.[59] Schwartz et al.[78] suggested that direct damage to T-cells by the CMV resulting in interference with cellular immunity could account for persistence of CMV. Selgrade et al.[75] proposed that CMV could interfere with macrophage function resulting in dysfunction of cellular immunity. Other theories proposed included CMV antigen - antibody complex formation resulting in CMV-immune-complex changes in tissues such as the glomeruli.[59,76,77]

Latent CMV is activated in conjunction with pregnancy, transplantation plus immunosuppression, perfusion and multiple transfusions, and disseminated malignancies. All of these conditions are accompanied by the presence of a homograft or homograft analogy. Either or both of these may contribute to activation of latent CMV.[59] The work of Wu et al.[79] Olding et al.[80] and Cheung et al.[81] suggested immunologic basis for activation of latent CMV. The cellular response to the presence of foreign antigens could activate CMV.

The persistency, latency and ability of CMV infection to reactivate pose difficult diagnostic problems. It is

often difficult to be certain whether a clinical CMV infection is primary or reactivated except in infants who are congenitally infected. This is because the CMV antibody level may fluctuate so much that even though the test might be said to be negative some months back, there is no assurance that there was no latent CMV infection. Unfortunately the commonly used CF test to detect the virus is not sensitive enough to detect all the seropositive individuals. Therefore, when it is vital to detect even low levels of CMV antibodies, CF test may not be the best. All these properties are shared by other herpes viruses.

3.3 Cytomegalovirus Infection Among Renal Transplant Patients

In an attempt to prevent allograft rejection, renal transplant patients are usually treated with immunosuppressive agents such as corticosteroids and azathioprine, or other agents. Almost all of these patients so treated are immunosuppressed to varying degrees.

Both in retrospective and prospective studies, Kanich et al.[11] showed that cytomegalovirus infection was associated with the use of immunosuppressive agents. Also, the work of Hedley-Whyte and Craighead[12] demonstrated that the prevalence of CMV infection has increased among transplanted patients since the introduction of immunosuppressive agents. Although the sample sizes were small in these studies, the results were clinically impressive.

Spencer et al.[19] observed that 92% of seropositive renal transplant individuals had significant antibody rises post-transplant. The corresponding figures for the seronegative patients (primary infection or seroconversion) was 70%. The authors recognized, however, that the number of the seronegative patients was too small (n=14) to make a meaningful comparison with the seropositive (n=86) patients. These data support the very frequent occurrence of CMV among renal transplant patients reported by others. Numerous studies[10,12-19] strengthen the conclusion that the prevalence of this virus, and the clinically manifested effects of the CMV infection are in excess of that expected in the general population.

If this virus was always asymptomatic and inactive, provoking neither morbidity nor mortality, studying its prevalence, incidence and natural history among renal transplant patients would have been nothing more than an academic exercise. However, this is not always the case. There are many well documented cases of CMV associated pneumonia.[10-12,14,17,18] These patients can be very sick. Usually they excrete the virus not only in their urine but also through the respiratory tract.

With CMV viraemia and/or pneumonia, hepatic dysfunction is not unusual.[15,27] Luby et al.[27] observed that 20 out of 44 (45.4%) of post-transplant patients who

had fourfold rise in CMV antibody titer had biochemical evidence of hepatitis. Similar observations have been reported by other investigators.[13,19] Azathioprine is known to cause hepatic dysfunction. It can be difficult to ascertain whether the hepatic dysfunction is due to azathioprine or CMV infection.

Febrile episodes of unexplained cause associated with at least a fourfold rise in CMV antibody titer makes this virus an etiological suspect. This type of febrile illness in conjunction with pneumonitis and impaired hepatic and renal function associated with at least a fourfold rise in CMV antibody titer has occured in an epidemic proportion (Coulson et al.) among renal transplant patients.[16] In this study, the virus was isolated either from the urine or from the throat swab or both.

There has been a suspicion that CMV infection is associated with some rejection episodes.[5,15] Although no causal relationship has been established between CMV infection and renal transplant rejection, this association, along with morbidity alluded to above, makes CMV an important virus in renal transplantation.

Mortality among renal transplant patients has been associated with heavy CMV infection in occasional instances. The only organism detected in a particular case was CMV.[12] The lungs and kidneys were heavily infected with CMV even though the patient was screened free of pneumonia 6 days prior

to this (post-transplant) fatal febrile illness. It may, therefore, be concluded that among renal transplant patients CMV infection may be associated not only with significant morbidity but also mortality.

3.4 Sources of Cytomegalovirus Post-Transplant

A knowledge of the source of the virus is helpful in the study of the incidence and morbidity this virus infection inflicts on the patients. There are speculations and experimental studies which point to different directions as to the source of CMV in a post-transplant CMV infection.[28,29] Ho et al.[28] showed that there was a significant correlation between the development of CMV infection in the recipient and seropositivity of the donor (P=.03) particularly when the recipient is seronegative (P=.02). The investigators concluded that the primary infection and CMV disease may be caused by the latent CMV in the transplanted kidney.

On the other hand, Armstrong et al.[29] presented data incriminating banked blood as a source of CMV. Their findings were in keeping with previous reports.[30,31] Re-activation of latent CMV in an immunosuppressed individual has been proposed by other investigators.

At the present time, it can be said that the source of primary CMV infection in a post-transplant patient is uncertain. The implication of transplanted kidney as a source of CMV are far reaching. It may become important to

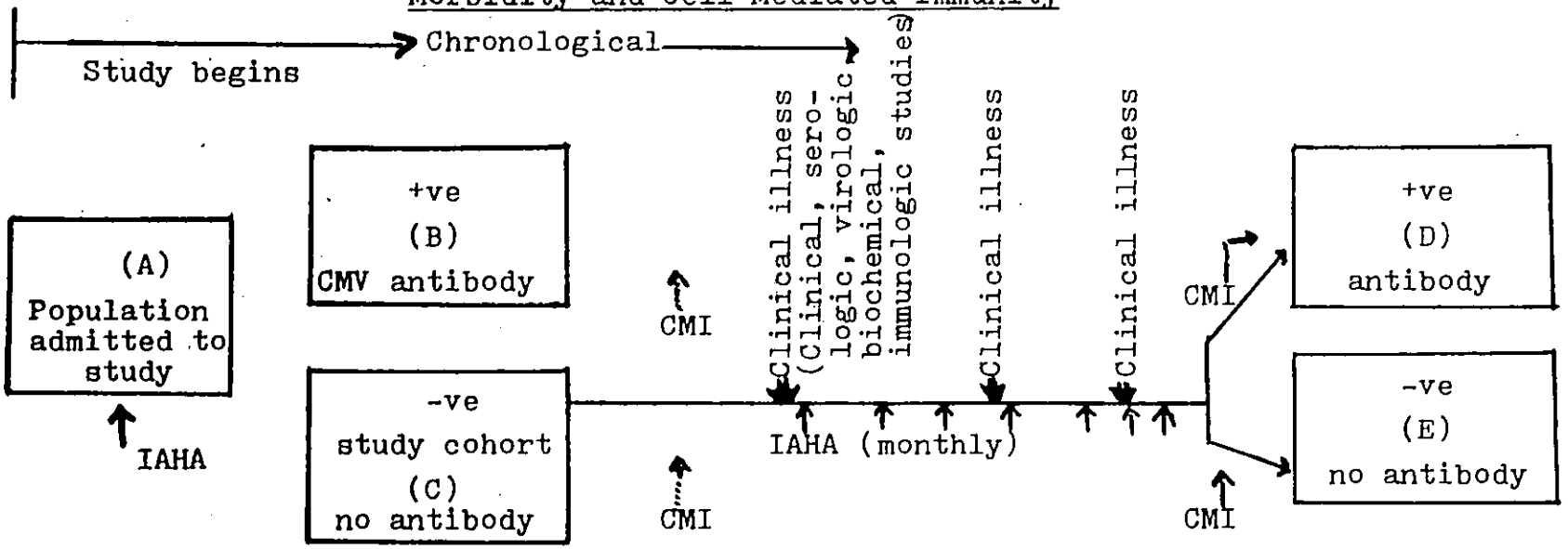
screen donors (as they are screened for hepatitis B) before such kidneys are accepted as fit for transplant. This may be done along with tissue typing. However, the clinical importance of CMV must be better understood, and the suspicion that the kidney may be a source of CMV confirmed before screening donors for CMV.

In a double blind controlled clinical trial, Cheeseman et al.[93] has shown that interferon may be effective in modifying CMV infection in renal transplant patients. Although their results are preliminary, it points to the fact that the treatment of CMV infection may reduce rejection episodes.

CHAPTER FOUR

FIGURE 4.1

Overall Experimental Design to Study Incidence, Morbidity and Cell-Mediated Immunity



KEY:

(1) IAHA = Immune Adherence Hemagglutination Test

(2) CMI = cell-mediated immunity, measured at strategic points in time for any change in the cellular immunity of patients (sample)

(3) = monthly measurement of CMV antibodies to study incidence

(4) = periodic clinical illness investigated fully for role of CMV in the morbidity experienced by the patient

(5.) Analytic Strategy:

(a) Compare morbidity in B v D; D v E; B v E

(b) Compare cellular immunity in B v C; C v D; C v E and D v E

(c) $\frac{D}{C}$ x 1000 = Incidence (over 18 months)

CHAPTER FOUR

GENERAL STUDY DESIGN, SAMPLE SIZE, STUDY POPULATION AND ADMINISTRATIVE STRATEGIES FOR THE STUDY

4.1 Study Design

The overall study design is as laid out in Figure 4.1 (page 27). The patients who meet the admission criteria (see section 4.4) will be separated serologically into two groups (Group B - CMV antibody positive and Group C - CMV antibody negative). The CMV antibody negative Group C constitutes an inception cohort for the study of incidence. The serologic test (IAHA) will be done on the sera of these patients monthly for 18 months. The proportion that converts to positive (D) out of the original study cohort with CMV negative will allow estimates to be made of the incidence of CMV among renal transplant and hemodialysis patients.

Concurrently with the monthly study of incidence will be a careful reporting (blind assessment as detailed in Chapter Seven) of any morbid state among these patients. If there is clinical illness (defined in Chapter Seven) that can be attributable to CMV, the patient will be investigated extensively (as indicated in the diagram) for evidence of CMV that may account for or be associated with the morbid

state. To that end, any fever, arthralgia, respiratory symptoms, malaise or any evidence of rejection episode will form the morbid factor for which the patient must be thoroughly investigated.

The cell-mediated immunity status of a sample of patients in Groups B and C would be determined by lymphocyte blastogenesis (lymphocyte stimulations tests). The tests would be done at the outset (having separated the patients into positive or negative for CMV antibodies); during any acute illness and at the conclusion of the study.

4.2.1 Sample Size Determination

In clinical or biological research, often the population of primary interest is so large that only a sample from the population is studied. The findings from the study of a sample which is chosen appropriately can be assumed to apply to other members of the population from which the sample comes. This statement assumes a proper, unbiased sampling technique. There is always the problem of how confident is the investigator when he assumes that the findings from studying a sample apply to the whole population. If the whole population can be studied, then the confidence is 100%. Whenever a sample is studied, the confidence that the findings apply to the whole population is always less than 100%. The bigger the sample size, the closer the findings to the true situation with the total

population, and the greater the confidence that the findings can be generalized.

Similarly, if there are two populations to be compared with respect to differences in the rate of a certain event taking place in these populations, samples can be drawn from these two populations. The question can be asked "Is there any real difference between these two groups with respect to the event under investigations?" Again, the larger the sample sizes are, the more confident the investigator is (given the observed difference) that there are real differences between the two populations with respect to the event under investigation. On the other hand, if the samples are small, the observed difference may be due to chance alone.

It is, therefore, important to estimate the sample size needed to be able to detect significant differences with a specified degree of confidence. This must be done before the commencement of the study.

In this study, there are three components viz:

(a) incidence study,

(b) study of cell-mediated immunity, and

(c) morbidity/mortality study.

These components need sample size determination in advance of the study.

4.2.2 Basic Statistical Definitions and Principles

(a) Normal Distribution

This is the well-known bell-shaped curve. It is a continuous symmetrical curve with both tails tending to infinity. Because there are varying standard deviations and means when different experiments are performed, there are many normal curves as shown in Figure 4.2 (a) on page 32.

(b) Standard (or Unit) Normal Distribution

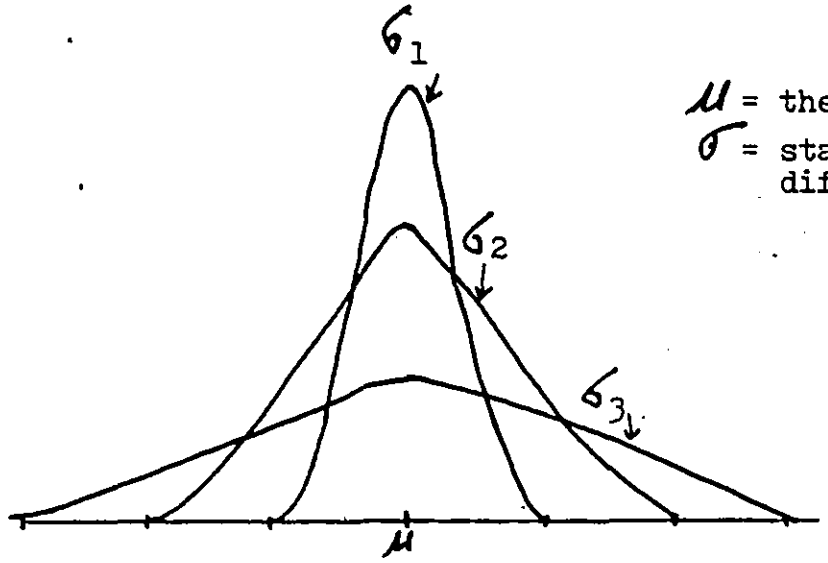
Of the many normal distributions mentioned in (a) above, the curve that is specified by standard deviation = 1, mean = 0 and area under the curve = 1 is known as standard normal distribution. The curve is as shown in Figure 4.2 (b) on page 32.

The observed data from any particular normal distribution must be standardized. The standardization is achieved by converting the observed values to a standard score (Z) whose mean = 0 and standard deviation = 1. All normally distributed data are, therefore, referred to this standardized Z value as shown in Figure 4.2 (c) on page 33.

(c) Alpha (α) and Beta (β) Levels

In any research endeavour, decisions are made as to whether the data that are available disprove or not, the prevailing opinion (null hypothesis). In making such

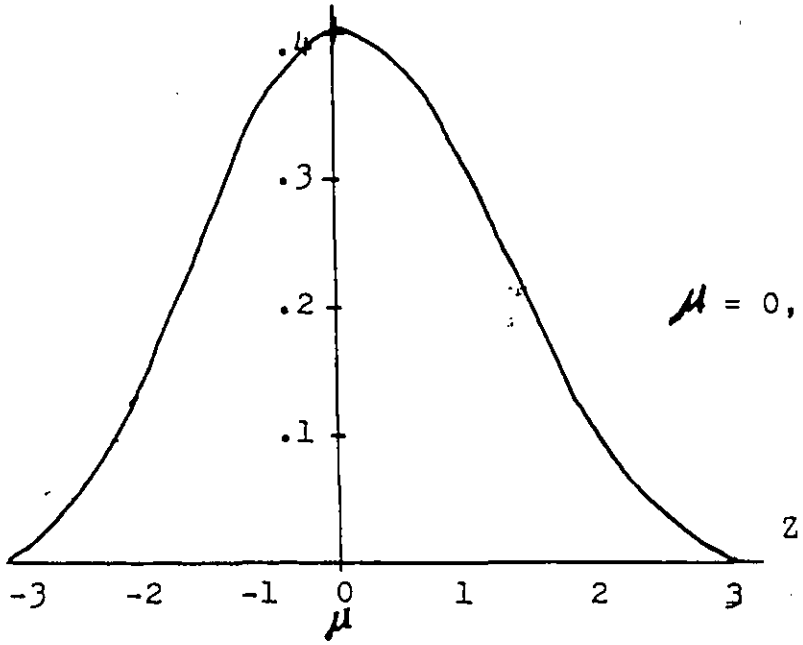
FIGURE 4.2 (a)



μ = the same for all but
σ = standard deviation differs

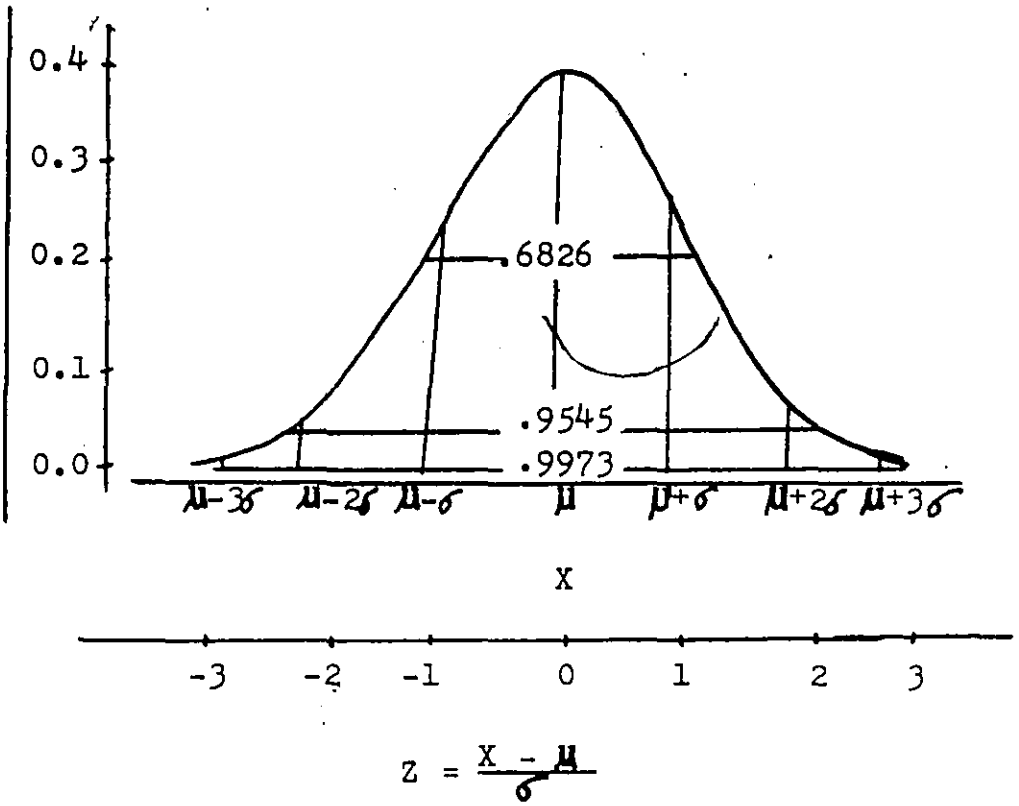
$\sigma_1 < \sigma_2 < \sigma_3$ where σ is a measure of spread.

FIGURE 4.2 (b)



$\mu = 0, \quad \sigma = 1$

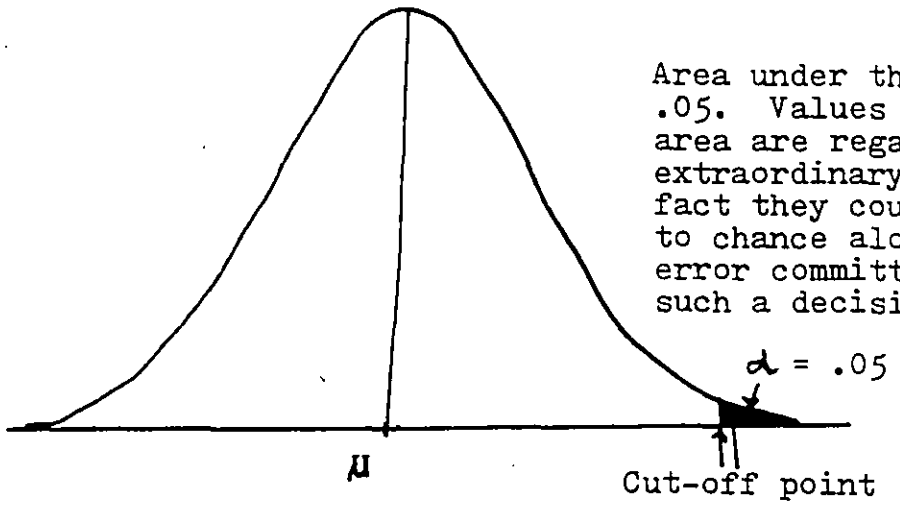
FIGURE 4.2 (c)



$$Z = \frac{X - \mu}{\sigma}$$

Thus Z = 2 means X can be located 2σ distal to the mean

FIGURE 4.2 (d)



Area under this curve = .05. Values within this area are regarded as extraordinary when in fact they could be due to chance alone. Type I error committed in making such a decision.

FIGURE 4.2 (e)

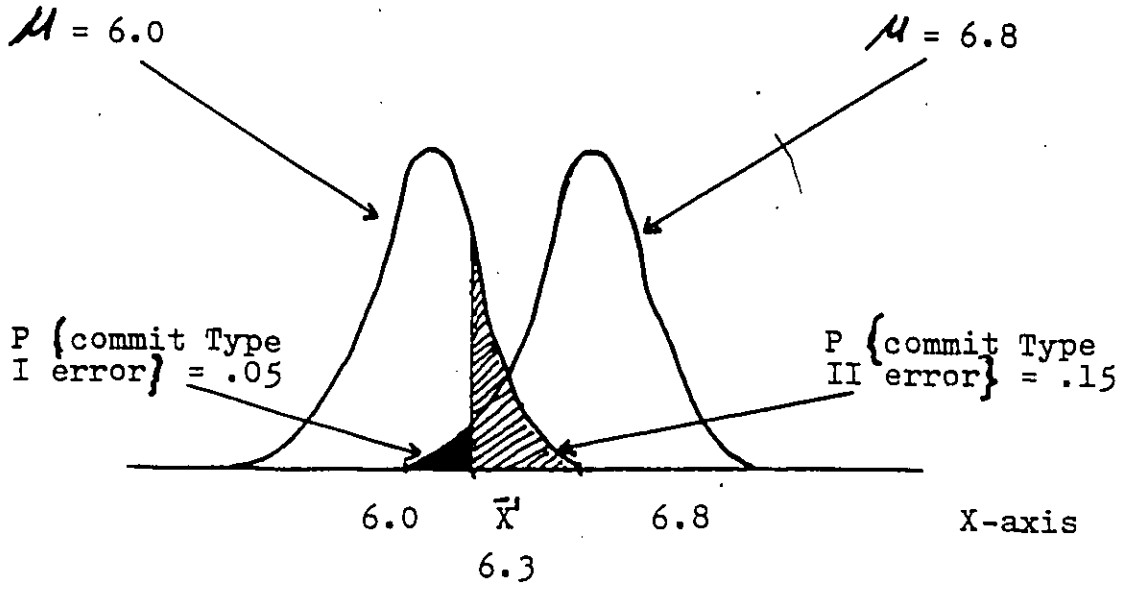
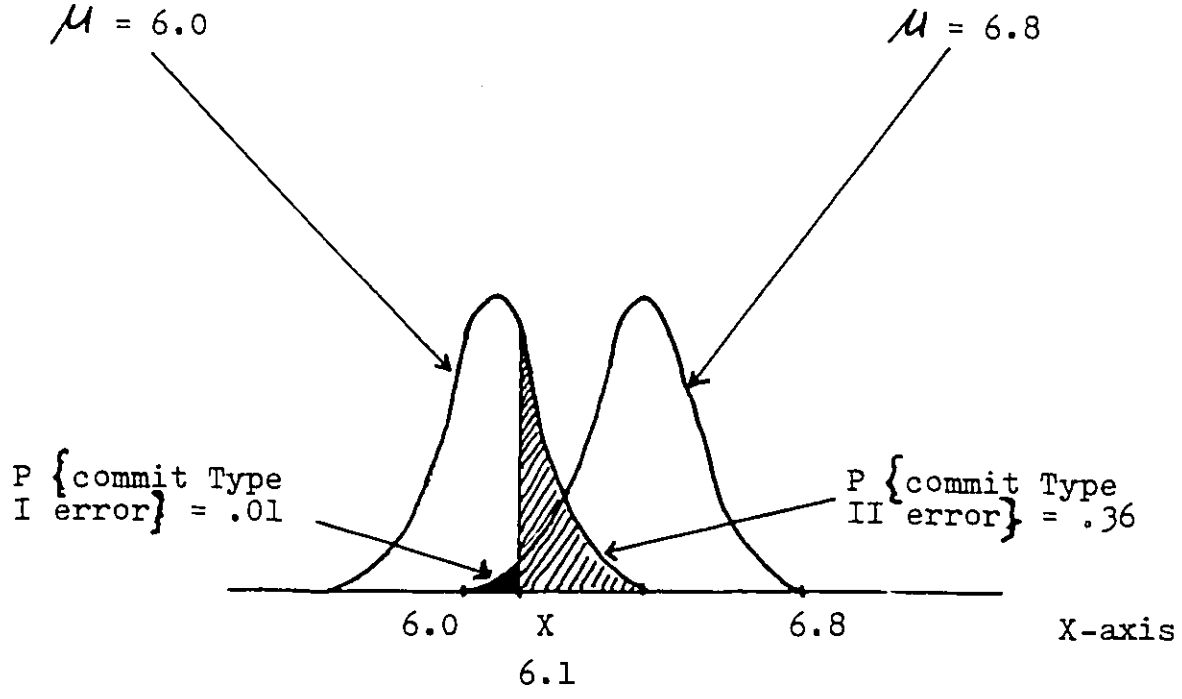


FIGURE 4.2 (f)



decisions, the researcher is aware that he/she could be wrong. He decides in advance, the chance of his making a wrong decision. That chance is the level of significance and is denoted by α . Most researchers usually use $\alpha = .05$ or $\alpha = .01$ (5% or 1% chance of making incorrect decision). This is also called type I or α error (see Figure 4.2 (d) on page 33.

On the other hand, the researcher may fail to decide that there is a significant difference between the null hypothesis and his findings. He then decides there is no difference when, in fact, there is. Such an error is called type II or β error. Decreasing one type of error increases the other type. Hence, depending on the nature of the problem the level of significance is chosen bearing the chance of β error in mind. Both the α and β levels are important in calculating the sample size required for any study.

4.2.3 (a) Sample Size for Incidence Study

In order to calculate the required sample size, the investigator must decide on two specifications: (i) tolerance range and (ii) confidence level. The tolerance range refers to the amount of error the investigator is willing to accept. It is known from the literature (Spencer et al.)[19], (Rifkind et al.)[10] that about 60-70% of the CMV seronegative renal transplant patients will develop antibodies to CMV. Suppose

there are 165 seronegative renal transplant patients to study. Given that 60-70% seroconvert (average = 65%), then the incidence rate estimated = 65/100 = 0.65. Let the number that seroconvert be r, n = total seronegative patients.

$\frac{r}{n} = \frac{r}{165}$ has $r \sim$ Binom. (165, 0.65). If $\frac{r}{165}$ is approximately $\sim N\left[.65, \frac{.65\ (1 - .65)}{154}\right] = \left[r, \frac{P\ (1 - P)}{n}\right]$

where P is the proportion that seroconvert, then the 95% confidence interval for the true value of $\overline{\Lambda}$, the probability of seroconversion

$$= \frac{r}{n} \pm Z_{1-\frac{\alpha}{2}} \sqrt{\frac{P\ (1 - P)}{n}} \quad \text{(two-sided test)}$$

$$= \frac{r}{n} \pm 1.96 \sqrt{\frac{0.65\ (1 - 0.65)}{165}}$$

Therefore, the width of the confidence interval (C.I.) =

$$= 2 \times 1.96 \sqrt{\frac{.65 \times .35}{165}}$$

$$= 3.92 \times .037132 = 0.145 = 14.5\%.$$

The width of the confidence interval = 14.5%. The confidence interval itself = 65 ± 7.25

= 57.75 to 72.25

only if the observed conversion ($\hat{\Lambda}$) is 65%. It is the width of the confidence interval which is most important and not the confidence interval itself.

This implies that with the estimated available 165 seronegative renal patients, the width of our 95% confidence limit is almost 15%. If a narrower confidence interval was desired, this would mean an increase in the sample size is required. For example, if the investigator wants only a 10% range (± 5%), then the sample size needed would be increased. Thus:

$$10\% = 2 \times 1.96 \sqrt{\frac{0.65 \times .35}{n}}$$

$$0.1^2 n = 3.92 \times .65 \times .35 = 3.496$$

$$n = 3.496 \div 0.01 = 349.6 \simeq 350$$

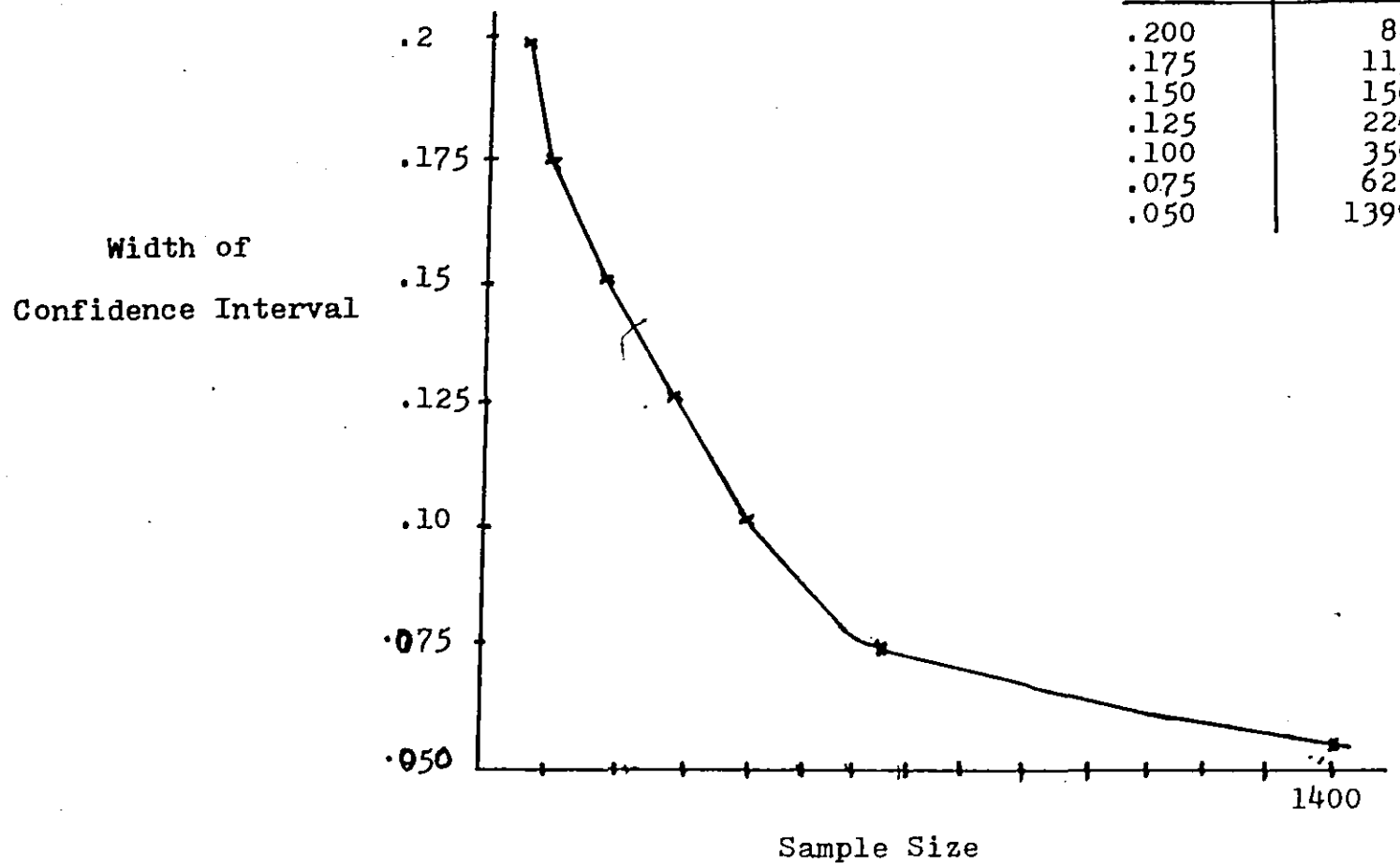
We, therefore, need for a 95% confidence interval of 10% approximately double the sample size for a confidence interval of 15%.

Similarly, calculations can be made for the confidence intervals of width 20%, 17.5%, 12.5%, 7.5%, and 5%. The corresponding sample sizes are shown below:

Width	Sample Size (n)
0.200	88
0.175	115
0.150	156
0.125	224
0.100	350
0.075	622
0.050	1398

FIGURE 4.3

Width	Sample Size
.200	88
.175	115
.150	156
.125	224
.100	350
.075	622
.050	1399



This width of 95% confidence interval is plotted against the corresponding sample size required as shown in the figure. For the present study, a width of approximately 15% is expected.

4.2.3 (b) Sample Size for Morbidity/Mortality Studies

Sample size calculations in morbidity/mortality studies associated with CMV among renal transplant patients is rendered difficult because there are rare previous reports that address the issues of sample size. The very few reports that have data on morbidity among CMV seropositive and seronegative patients are rendered of little use because of very small sample sizes.[13,14] There are instances where the number of seronegative patients that seroconvert and experience morbidity were reported without indicating the total number of seronegative patients under investigation. This sample size calculated is, therefore, based on little information.

Various morbid factors may be used to calculate sample sizes and the largest sample size calculated is used to conduct the studies. For example, in this study, morbid factors such as fever, arthralgia, rejection episodes may be used. The proportion of seropositive and those of seronegative that have the morbid factor is then compared say P_2 = proportion that has morbid factor among the seropositive, and P_1 = the proportion of the seronegative

that experience the same morbidity are then compared for differences.

This sample size calculation is based on the reports of Andersen et al. who reported 30% morbidity among renal transplant patients who developed primary CMV infection. Betts et al. also reported 34% morbidity among his similar groups of patients. Fiala reported 43% morbidity among those who became CMV seroconverted. The average of these reports = 35.67 = 35%.

In calculating sample size, both the α and β levels must be predetermined. These two values fix the risk the investigator is prepared to take. In general, the larger the sample size the less the risk of erroneaous conclusions.

If then the proportion of the seropositive renal transplant patients that become morbid post-transplant is 35% and if one wants to be able to detect differences of 50%, then the proportion of seronegative renal transplant patients showing clinical morbidity will be 50% of 35 = 15% = P_1 where P_2 = 35%.

Sample size for studies that involve proportions may be calculated from the given proportions or read off directly from tables prepared for such studies. The general formula for calculating sample sizes where proportion are given can be represented thus:

N = 2n where N = total number for both control and test samples.

$$n = \left[\frac{Z_{-\frac{\alpha}{2}} \sqrt{2\bar{P}\bar{Q}} - Z_{1-\beta} \sqrt{P_1Q_1 + P_2Q_2}}{(P_2 - P_1)} \right]^2$$

For this study, n has been read off from tables prepared by J. Fleiss.[83]

Let $\alpha = .05$ and $\beta = .10$

$P_1 = .15$, $P_2 = .35$ (two-sided test)

N = 2n = 232, n = 116

2n = 194 = 2 x 97 for one-sided test.

Our primary interest here is the one-sided test. Assuming 50% seroconversion, then $0.5n = 97$

$n = 194$

Hence we need 194 patients who are seronegative, 50% of them would seroconvert = 97. With 97 seroconverted and with the specifications of α and β levels, significant differences between seronegative and seropositive individuals (in terms of morbidity) would be detected.

Since we suspect from the literature that the original seropositive patients tends to reactivate more frequently but have less morbidity, it is assumed that the degree of morbidity in the originally seropositive individuals will be somewhere between what the seroconverted and what the always seronegative experience. Therefore, sample size

that takes into account the seroconverted and seronegatives need will be adequate for the always seropositive individuals. Decision on sample size: Study 194 seronegative and 97 seropositive individuals. But because of constraints on the number of patients that are available (certainly not less than calculated number) for the study, all the seropositive and seronegative patients. Because = .05, the 95% confidence limit can be calculated.

4.2.3 (c) Sample Size Determination for Cell-Mediated Immunity Study

This study is based on radioactive counting. The mean count for CMV specific antigen stimulated lymphocytes from an individual will be compared with the mean counts for lymphocytes (without antigen) from the same individual. The ratio of mean count for antigen stimulated lymphocytes over the mean count for the lymphocyte without antigen (stimulation index) is greater than 3.[35] Since we are dealing with means, a Student t-distribution is involved. In deciding on sample size under this circumstance, the concern is to have sufficient number so that the distribution approaches that of normal distribution. This follows the central limit theorem which states that the sampling distribution of means is approximately a normal curve, regardless of the shape of the parent population distribution provided the sample size is large enough.

In order to estimate the sample size, an estimate of the expected variance of the counts and the differences

in the counts between the two groups - control and experiment must be determined. The data of Waner et al.[65] gave the mean counts and standard error of the mean (S.E.M.)[91] on their study subjects. Their data is as presented in Table 4.1.(page 44)

The standard error of the mean for the control appears quite different from that for the test (CMV Ag). However, the S.E.M. may be proportional to the radioactive count. By dividing the S.E.M. for the test by the stimulation index and taking their natural logs, the S.E.M. for both the control and the test are comparable as shown in Table 4.2.

TABLE 4.2

	Control	CMV Positive
log SEM =	.0718	.0373
	.0778	.0668
	.0842	.0735
	.0422	.0866
	.0525	.0322
	.0067	.0220
	.0980	.0471
	.0880	.0346
	.0697	.0639
	.0366	.0310
	$\bar{X}_1$ = .0627	$\bar{X}_2$ = .0523

Therefore, the S.E.M. for both sides are about equal. This S.E.M. represents the within person variation.

TABLE 4.1

Lymphocyte Response to Control and CMV Ag

Donor	Baseline*	Control Ag	CMV Ag
R.C.	4459 ± 386	5559 ± 399	181409 ± 6761 (32.63)**
S.P.	1879 ± 95	1980 ± 154	65563 ± 4381 (32.10)
E.M.	5353 ± 101	5818 ± 490	143641 ± 10555 (24.69)
M.T.	2844 ± 222	4886 ± 206	116032 ± 10050 (23.74)
N.R.	1892 ± 104	2591 ± 136	40040 ± 1289 (16.61)
T.W.	2603 ± 147	2377 ± 16	34845 ± 768 (14.66)
G.K.	2184 ± 93	1622 ± 159	23676 ± 1114 (14.60)
J.W.	7723 ± 575	5172 ± 455	58490 ± 2026 (11.30)
L.K.	1491 ± 48	1579 ± 110	10643 ± 680 (6.74)
K.M.	1582 ± 83	7820 ± 286	48539 ± 1503 (6.18)

* Cells incubated only with medium

** Numbers in parentheses show SI.

For an unpaired sample, the more important variation is between patients. For this, a log transformation on the mean counts could be obtained. The advantage of log transformation is that the stimulation index will be easy to express in log form since it is a ratio. Ordinarily the stimulation index, S.I. = $\frac{\bar{X}_2}{\bar{X}_1}$ where $\bar{X}_2$ is the mean count for the lymphocyte + CMV antigen and $\bar{X}_1$ is the mean count for lymphocyte without antigen.

S.I. = $3 = \frac{\bar{X}_2}{\bar{X}_1} = \log_e \bar{X}_2 - \log_e \bar{X}_1$ such that the problem becomes the differences between log means. What must be known is variance log $\bar{X}$. Taking the natural logs of the mean counts for lymphocytes with control antigen and CMV antigen groups, $\log_e \bar{X}_1 = 8.13$ and $\log_e \bar{X}_2 = 10.8904$

$$\text{Var } (\log_e \bar{X}_1) = 0.3513 = \sigma_1^2 \text{ (calculated)}$$

$$\text{Var } (\log_e \bar{X}_2) = 0.7405 = \sigma_2^2 \text{ (calculated)}$$

Taking the root mean square of these variances

$$\sigma^2 = \sqrt{\frac{\sigma_1^2 + \sigma_2^2}{2}}$$

$$= \sqrt{\frac{0.3513^2 + 0.7405^2}{2}}$$

$$= 0.57955$$

$$\therefore \sigma = \text{S.D.} = \sqrt{.57955} = 0.76128$$

Now, the minimum significant difference of S.I. to be detected be set at 3. Take natural $\log_e 3 = 1.0986 = \Delta$ The ratio $\frac{\Delta}{\sigma}$ is a variable which together with the fix α and β levels determine the sample size. For a given α and β levels, the sample size that corresponds to the ratio $\frac{\Delta}{\sigma}$ can be read from standard tables. In this case,

$$\frac{\Delta}{\sigma} = \frac{1.0986}{0.76128} = 1.443$$

Set $\alpha = .05, \beta = .05$ (one-sided test), the ratio $\frac{\Delta}{\sigma} = 1.443$, the sample size, n = 12 from table.[90] Therefore, we need 12 seropositive, and 12 seronegative renal transplant patients for the lymphocyte stimulation studies.

Decision: Study 15 seropositive and 15 seronegative patients.

It is intended that patients who are known to have CMV antibody (always positive) from the inception of the study, those who become seroconverted and those who remain seronegative will have their lymphocytes studied. It is easy to randomly select 15 seropositive individuals. But it is difficult to predict who among the seronegatives will become positive and who would remain negative. Therefore, the size of the seronegative individuals among whom 15 seroconversion and 15 persistent seronegative requires special consideration.

The proportion that is expected to seroconvert will determine how many seronegatives must be followed in order to find 15 persistent seronegative and 15 seroconversion.

Assuming 60% seroconversion, then 40% remains seronegative. Then .4n = 15 if n = total seronegative required and 15 is the sample size for each of the groups. Therefore, n = 38 patients. But the level of confidence that this 60% seroconversion gives is essential. The problem is best appreciated if different proportions that may seroconvert is tabulated against the confidence intervals around the proportions. For example, when 40%, 50%, 60%, 70% seroconvert, the 95% confidence limits are calculated as shown in Table 4.3 given a sample size of 75. P = proportion serconvert, N = sample size, C.I. = confidence interval, +ve = CMV positive, -ve = CMV persistently negative.

Table 4.3 (page 48), therefore, demonstrates the fact that if it happens that there is 70% seroconversion, a sample size of 75 seronegative individuals will be desirable to have about 15 persistent seronegative individuals.
Decision: Follow 75 seronegative individuals randomly selected among the study population.

4.3 Population for the Study

For this study there are two alternative populations. The study may be limited to one renal transplant and dialysis programme such as the Hamilton Regional Renal programme. On the other hand, the study may draw patients from Hamilton Regional Renal programme plus the group of seven hospitals in Toronto that undertake renal transplant and dialysis.

TABLE 4.3

P	N	95% C.I.	CMV +ve	CMV -ve
40%	75	11%	22 - 38	37 - 54
50%	75	11	29 - 45	30 - 45
60%	75	11	37 - 53	22 - 38
70%	75	10	45 - 60	15 - 30

The study then becomes multicenter. There are advantages and disadvantages for either method.

The study that involves only one center (Hamilton Regional Renal Unit) has a unique advantage in that the diagnostic criteria, indications for renal transplant and hemodialysis would be the same for all patients. In the study of morbidity and mortality attributable to CMV infection, measurements such as BUN, creatinine, SGOT, SGGT etc. would probably have less variations than when done in different centers. This is because the method used would be uniform. Also, if each test can be done by the same technologist, the interobserver variation would be elimination. Studying the Hamilton Regional Renal Programme alone has an added advantage of ease of administration due to the fact that specimens for test come from the same locality and the tests results are obtained promptly. It would also be economical.

The main disadvantage of using single programme for the study is that there may not be enough patients to study based on the minimum number required as calculated above (section 4.2). The study may have to be prolonged. In the Hamilton Regional Programme, about 25 patients are being transplanted annually. With the existing 60 transplant patients, the study will take about 5 years to complete. Unless major changes take place in the management of renal

transplant patients, this duration would not matter much to the results.

To combine patients from both Hamilton and Toronto group of hospitals would enable the study to be completed in a short period of time. The data available indicates that there are 180 transplant patients per year from both Hamilton and Toronto. In 2 years (?1979 + 1980), there will be approximately 360 patients. However, because of many sources of variations and difficulty in administration, using the single center, Hamilton Regional Renal programme, is preferred. Therefore, this study would be based on the patients in Renal Transplant and dialysis in the Region of Hamilton-Wentworth, St. Catharines and Kitchener-Waterloo.

4.4 (i) Criteria for Admission into the Study

1. Patients with end-stage renal disease (as defined in section 2.1) who require renal transplantation or hemodialysis.

2. Patients who are either from Hamilton or any of the other hospitals listed under Hamilton Regional Renal Programme.

3. Patients who freely sign written informed consent.

4.4 (ii) Exclusion Criteria

Patients who are likely to move out of the Hamilton area during the study period. The chances of patients

moving in or out of the region to other centers are small; given the seriousness and nature of their chronic ill health. Once established in hemodialysis unit, they tend to stay on in the unit they know already. Therefore, such patients who would move will be very small in number.

4.5.1 Administrative Strategies for the Management of Study

The importance of good administrative mechanism for any study cannot be overemphasized. Duty specification and preplanned methods of flow of specimen movements, specimen results and data management are important components of a good study. For this study, all tests will be done at St. Joseph's Hospital in Hamilton, except lymphocyte blastogenesis which would be done at the McMaster University Immunology Laboratory. X-ray films will be read by one radiologist. Every effort will be made to ensure that one technologist processes all IAHA, biochemical determinations unless it is done on an emergency basis.

4.5.2 Research Team

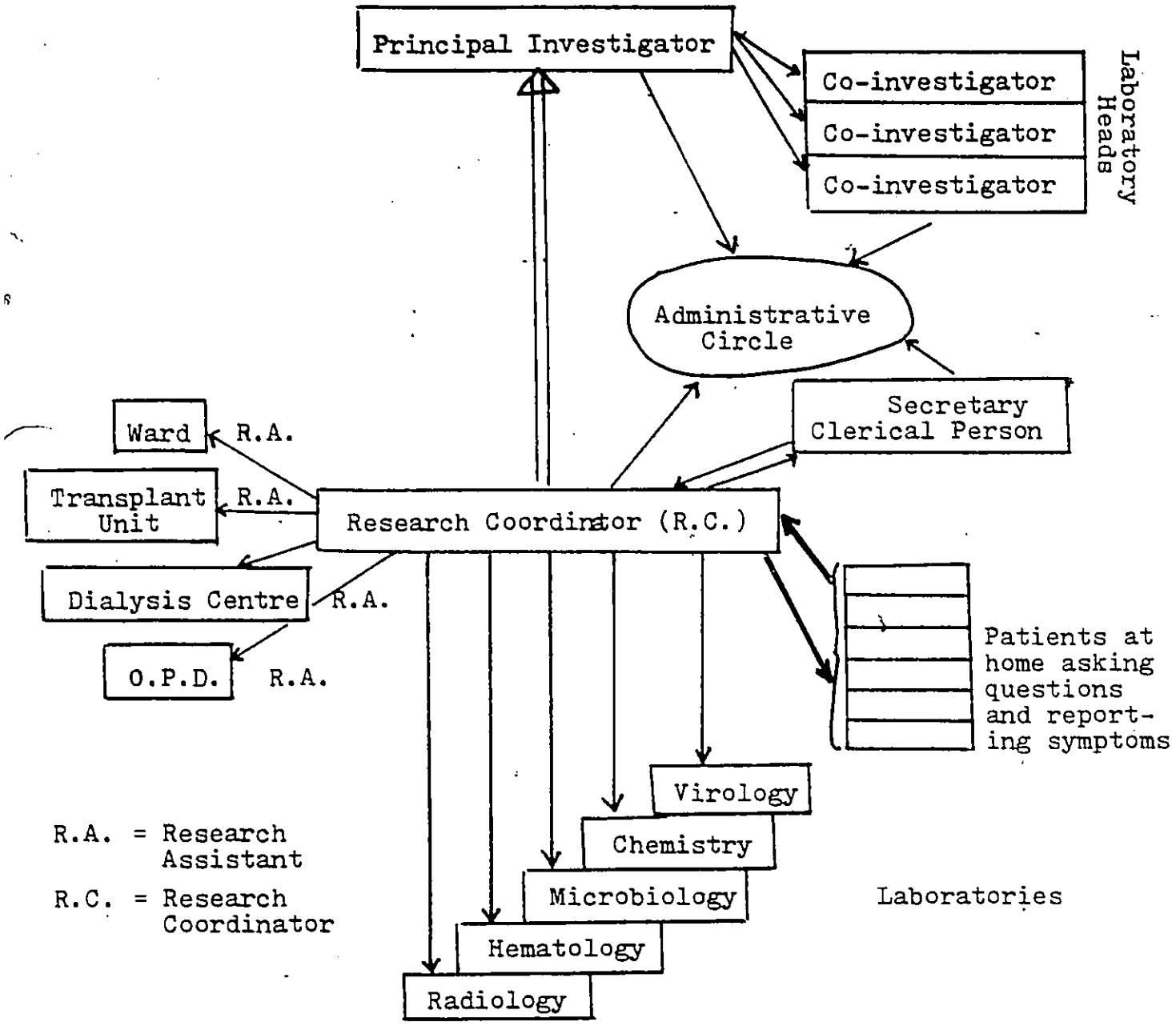
(a) There will be a principal investigator who has overall responsibility for the study. His role is supervisory and coordinating. He directs the study and meets regularly with the Research Coordinator (R.C.), the co-investigators and laboratory team in case of any major problems.

The co-investigators will be laboratory department heads who are competent investigators, interested in the study, who will be in the administrative circle meeting. They will bring forth their suggestions and criticisms. A clinical nephrologist will be included as either co-investigator or principal investigator.

There will be research assistants in the wards where these patients might be cared for, in the dialysis units, transplant unit and outpatient department. Their roles are to gather and/or obtain specimens from the patients, relate positively to these patients, and hand over the specimens and laboratory results to the R.C. The research co-ordinator is most important. This is a full-time job for the period of the study. The R.C. prepares the list of all patients in the study, their addresses and telephone numbers. He prepares the flow sheets for data management; he is freely available for the patients' questions and reports of symptoms. He submits study specimens to the laboratories and he relates to technologists regarding the studies being done on the specimens. He discusses the problems that might arise. He relates to the research assistants and the principal investigator. He reports all data to the principal investigator. He attends all administrative circle meetings with the investigators where he reports the progress and problems regarding the study.

FIGURE 4.3

Administrative Scheme



A clerical/secretary person who has good experience in typing technical reports will be required. She will update all clinico-laboratory reports as well as the field reports. She will update the all-purpose flow sheet. She also keeps the stationery and the purchases of all the essential equipment. She has regular reports to R.C. about the data. She may attend the administrative circle meetings.

CHAPTER FIVE

INCIDENCE OF CMV AMONG RENAL TRANSPLANT AND DIALYSIS PATIENTS

5.1 (a) Why Study Incidence of a Disease?

The study of incidence enables us to anticipate the prevalence of the condition in the future. It also enables us to study new cases near their outset.

5.1 (b) Definitions

(i) Incidence rate may be defined as the number of new events (cases) occuring within a specified period of time relative to the total number of persons exposed to the risk during that period of time.

$$\text{Hence, incidence rate} = \frac{\text{Total no. of new cases within a period}}{\text{Total no. of persons exposed to risk}} \times 1000$$

(ii) Point prevalence rate may be defined as the total number of cases existing at a specific point in time, relative to the total number of persons exposed to the risk.

$$\text{Point prevalence rate} = \frac{\text{Total no. of cases at a time}}{\text{Total no. of persons at risk at that time}} \times 1000$$

The objective here is to know how many cases were in existence at a given point in time (eg. on a given day). Prevalence of a disease is determined by factors such as incidence rate, mortality rate, migration (in or out),

recovery rate, relapse rate and uncovering.

Annual death rate per 1000 =

$$\frac{\text{Total no. of deaths during a specified period of year}}{\text{No. of persons in the population at mid year}} \times 1000$$

If the death rate is high, though all other factors mentioned above are constant the incidence will be low. On the other hand if the death rate is low, the prevalence will be high. Migration refers to movement of subjects in or out of the study region. If many subjects who have the disease under investigation migrate away, the prevalence will be low unless account is taken of those who migrate out. The reverse is true for the immigration. The immigration of subjects who have the disease under investigation will affect the prevalence if the condition under investigation is more frequent among the immigrants than the original population under investigation. Immigration in this context refers to the movement of subjects into the region where other subjects are being observed, the immigrant being extra-subjects, not part of the original study population. Recovery rate will also affect the prevalence rate. If a significant proportion of the population develops the outcome of interest and recovered before measurements can be made, such individuals will be regarded as not having the outcome of interest. Those who recover will be regarded as negative. If the condition can relapse then the old cases that were silent

may be diagnosed as new cases. This would increase the proportion that is regarded as "new cases", and would affect the incidence rate. Uncovering implies that there are subjects with the disease who are not diagnosed possibly because of lack of adequate instruments. Certainly such a situation would reduce the prevalence rate.

These factors are also important in the study of incidence of any condition. Many of them apply in this study and are discussed (see below). The basic distinction between incidence rate and prevalence rate is that incidence describes the change in the number that are affected (eg. number of new cases of CMV antibody acquisition in a specified time) whereas prevalence describes the total burden of the condition at a given point in time (eg. the number of patients with CMV antibodies among a given patient population at a given point in time).

In this study, the patients who die, those who withdraw from the study, migration into and from transplant and dialysis as well as those who seroconvert but die before diagnosis is made are very relevant. Any of these factors will affect measurement of both the incidence and prevalence rates. It is assumed that these factors will not occur frequently. When a transplant patient goes on dialysis and later transplanted again, these events will be carefully documented. If those who die, or withdraw from the study

are removed from the analysis of the data, then the denominator will decrease, and the incidence will appear higher than it really is. On the other hand, if they are included in the analysis without modifications, they will tend to dilute the incidence rate by increasing the denominator. Therefore, it is important to take into account the length of time during which they were in the study.

Early after entering the study, some would die, withdraw, transfer to dialysis or migrate; others would die or withdraw late. It may be assumed that these events will take place randomly. Therefore, a mid year correction will be applicable. Thus the conversion rate is calculated by counting the seroconversion that actually occured during the specified time interval and adding an adjustment to them for withdrawals, deaths, etc. for the expected seroconversion for the half of the interval they were not being monitored.[85]

Thus, for the interval x to x + 1, let O_x denote the number under observation at the beginning of the interval, d_x the seroconversion during the interval, W_x the withdrawals during the interval and q_x the conversion rate for the interval. Now, the expected conversion among the withdrawals/dead/transfers for half the interval they were not monitored $(W_x)(q_x/2)$ must be added to d_x. Hence the adjusted seroconversion = $d_x + (W_x)(q_x/2)$ so that the conversion rate

$$q_x = \frac{d_x + (W_x)(q_x/2)}{0_x}$$

$$\text{or } q_x = \frac{d_x}{0_x - W_x/2}$$

This general principle holds for the previously listed factors that may affect the incidence of CMV among renal transplants and dialysis patients.

Most of the studies entitled "incidence"[15,27] of CMV among renal patients are strictly prevalence studies. There is very little in the literature about the natural history of CMV infection among renal transplant patients. The studies that are available cannot draw meaningful conclusions because of small sample sizes or poor experimental design. For example, Spencer[19] followed 27 seronegative patients prospectively for seroconversion; 70% became seropositive and 30% remain seronegative. In this study, the proportion that convert was 0.7. The proportion that remain seronegative = 0.3. If π is the true probability of seroconversion in the population from which this study sample was drawn, then:

$$\pi = \frac{r}{n} \pm Z_{1-\frac{\alpha}{2}} \sqrt{\frac{P(1-P)}{n}}$$

where r = the number that seroconvert, n = the number of patients studied, P = proportion that seroconvert, Z = area under standard normal curve, α = the sufficiently small chance (probability) that the statement is false. Applying this formula to Spencer's data and focusing on the width of the confidence interval

$$\left[2Z_{1-\frac{\alpha}{2}} \sqrt{\frac{P\,(1-P)}{n}}\right]$$

it turns out that the width of the confidence interval is

$$2 \times 1.96 \sqrt{\frac{.7 \times .3}{27}} = 34.6\%$$

= width of 95% confidence interval. This implies that the variation one can expect in the study of incidence of CMV among renal patients using a sample size of 27 seronegative patients is as wide as 34.6%. It is desirable to reduce this width much lower. The important determinant of the width (given that the true probability of seroconversion is constant) is the sample size. Using similar calculation, if he could triple his sample size (n = 81), then the width of that confidence interval would be reduced to 19.9 which is reasonable.

5.2 Principles Involved When Studying Incidence

(i) Sample Size: The size of the sample that is needed in studying incidence depends on the frequency of

of the disease under investigation. If the disease frequency is high, then the sample size needed is relatively small. On the contrary, if the disease is rare, and its frequency of occurrence is very low, then one needs a large sample size. Also, the duration of study of incidence is influenced by the frequency of the disease. If the frequency is low, then one would follow the study sample for a relatively long time for the outcome of interest to be present.

(ii) Some of the members of the study sample would have the outcome of interest (eg. CMV antibodies) before the inception of the study whereas some do not. When studying incidence, the interest is initially on those who do not have the outcome of interest at the outset. Therefore, the study sample must be tested in order to separate those who have the outcome already from those who do not.

(iii) The instrument for measurement to separate those who had outcome from those who do not must both be sensitive and specific. Sensitivity of a measure (test in this case) is defined epidemiologically as the percentage of those who have the disease, and are so indicated by the test.

Specificity of a test is defined epidemiologically as the percentage of those who do <u>not</u> have the disease and are so indicated by the test. This is illustrated in Table 5.1.

TABLE 5.1

Indexes to Evaluate the Accuracy of a Test or Diagnostic Examination

Sensitivity and Specificity

Test or Examination	Disease Present	Disease Absent	Predictive Value
Positive (Disease Probab. Present)	A (True Positive)	B (False Positive)	A/A + B = +ve P.V.
Negative (Disease Probab. Absent)	C (False Negative)	D (True Negative)	D/C + D = -ve P.V.
TOTAL	A + C	B + D	

Sensitivity = $\frac{A}{A + C} \times 100$

Specificity = $\frac{D}{B + D} \cdot 100$

+ve = positive

-ve = Negative

P.V. = Predictive Value

Adapted (and modified) from: Foundations of Epidemiology by A. Lilienfeld[45]

Another important concept introduced into the table is predictive value (P.V.) of a test. The predictive value may be positive or negative. Positive predictive value of a test may be defined as the percentage of positive results that classify those who have the disease correctly when the test is applied to a population containing both healthy and diseased subjects. Negative predictive value of a test may be defined as the percentage of negative results that classify those who do not have the disease correctly when the test is applied to a population containing both healthy and diseased subjects. True positive means the test is positive in individuals known with the disease. True negative means the test is negative in individuals known not to have the disease. Sensitivity, specificity and predictive value of tests can, therefore, be summarized thus:

(a) Sensitivity = $\frac{\text{True Positives}}{\text{True positive plus false negative}} = \frac{\text{True Positive}}{\text{All those with the disease}}$

(b) Specificity = $\frac{\text{True Negatives}}{\text{True negatives plus false positives}} = \frac{\text{True Negatives}}{\text{All those without disease}}$

(c) Positive Predictive Value = $\frac{\text{True Positives}}{\text{Total Positives}} = \frac{\text{True Positives}}{\text{All positive results}}$

(d) Negative Predictive Value = $\frac{\text{True Negatives}}{\text{Total Negatives}} = \frac{\text{True Negatives}}{\text{All negative results}}$

So far, the concepts described (sensitivity, specificity, and predictive values of a test) are epidemiologic. Sensitivity as defined above is completely different from the sense in which sensitivity is used with respect to a laboratory work. In the laboratory sense, sensitivity is a measure of how low a concentration of a substance the test can detect. For example, a test may be able to detect 0.05 mg/ml of a substance in the serum whereas another test may be able to detect 0.005 mg/ml of the same substance. The second test is, therefore, 10 times more sensitive than the first test.

The laboratory concept of specificity implies that the test is positive only when the condition for which the test is being performed is present. If, for example, the test is designed for diagnosing streptococcal infections, and if the patient has whooping cough or any other infection other than streptococcal infection, and the test is negative, then the test is specific for streptococcal infections. Both the sensitivity and specificity of tests employed in incidence study are important since the false positive, false negative rates will affect the incidence rates as determined by the tests. The laboratory concept of these terms apply in this incidence study.

(iv) In this study, the outcome of interest is antibody to cytomegalovirus. The test to be applied is

immune adherence hemagglutination test (IAHA). This is a serologic test fully discussed in section 5.5. This test will be used to separate out those, among the study patients, who do and who do not have outcome of interest (seropositive and seronegative respectively) at the inception of the study.

(v) Those who do not have outcome of interest (CMV antibody in this case) among the study patients constitute a cohort that will be followed with serial measurements (or observations) starting from a defined time and followed for a specified period of time (18 months). For the present study, those who are seronegative are assumed never to have had CMV infection in the past. These seronegative renal transplant and dialysis patients form a cohort that would be followed for the development of CMV antibodies (i.e. seroconversion).

(vi) In the study of incidence, the outcome of interest should have a known duration. The frequency of measurements to detect the development of CMV antibody will depend on how soon one may expect some of these seronegatives to become seropositive. Monthly measurements will be adopted for this study.

(vii) The time interval for serial measurements (or observations) of those who are seronegative must be frequent enough that those who seroconvert during the follow-

up period are not missed. In this study, measurement will be made frequently not because the antibodies will disappear, but because the rate of acquisition of the CMV infection among this high risk population is high.[10-18] In general, the outcome of interest must not be so short-lasting that the event disappears before measurement can be made. Alternatively, the time interval between measurements must not be so long that some of the events are missed. Either of these situations would lead to apparent low incidence rate. The CMV antibody lasts so long that frequency of measurement is unlikely to affect its incidence rate. Frequent measurements will, however, be useful in detecting any association between how soon a patient seroconverts and certain factors such as the primary diagnosis, duration of dialysis and source of kidney transplanted.

(viii) All members of the cohort under investigation must have equal chance to be exposed to the risk (CMV) i.e. the patients must have equal chance of acquiring CMV infection. This is important because if a proportion of the study patients does not get exposed, then the proportion that will become seroconverted may be falsely low. Even though all the members of the cohort are seronegative, there may be differences in their rates of acquiring this virus (eg. the type of renal lesion,[19] the duration of dialysis before renal transplant,[19] age,[23] sex,[19,32] etc.)

Therefore, it is important to take these factors into account in the analysis of the data.

5.3 (i) The Study Population

This consists of patients in Renal Transplant and Dialysis Programme in Hamilton-Wentworth Region, Saint Catharines, and Kitchener-Waterloo. All these patients are in one regional renal programme with the base at St. Joseph's Hospital, Hamilton, Ontario. Only those who have met the criteria for admission to the study as outlined in Chapter IV constitute the study population.

5.3 (ii) Incidence

This chapter pertains to the study of incidence of CMV among renal transplant and dialysis patients. Incidence has been defined in section 5.1 above. Incidence can be measured by at least four different ways:[32]

(a) by looking at compulsory reports for specific diseases,
(b) by looking at hospital data,
(c) by manipulating prevalence and duration data,
(d) by performing specific surveys.

The last method of studying incidence will be used for this study. The survey is in the form of testing serum from the study patients for antibody detection. The patient described as CMV seronegative in section 5.4 out of the total population admitted to the study (in section 5.3.1) will

be followed for acquisition of CMV antibodies. The proportion that will develop CMV antibodies (equivalent to primary CMV infection) within a specified period of time is the incidence of CMV in that study population.

5.4 Instruments for Measuring CMV Infection

The nature of CMV is such that when individuals come in contact with the virus, the infection is usually so mild that the subject does not know about it or that the infection is completely asymptomatic. Therefore, clinical observations cannot be relied upon in ascertaining who has had the infection in the past. In the acute phase of the infection, individuals usually excrete the virus in the urine and through the respiratory tract. Urine cultures and throat swabs are suitable as the means of screening those who have had the infection. These methods are suitable only in the early phase of the infection.

Advantage is taken of the fact that when anyone is infected by CMV, the body mounts a defense mechanism whereby specific CMV antibody is produced. This is the basis of immunity to the virus. The antibody persists in the body whether the primary infection was symptomatic or asymptomatic. Measurement of CMV antibody is, therefore, useful in ascertaining CMV infection. This is the method chosen for the study of incidence of CMV portion of this thesis. It involves serologic methods.

5.5 Serologic Tests

There are serologic tests that can be employed to detect antibodies in the serum of the subjects admitted to the study, eg.:

(a) Complement Fixation Test (CF)

(b) Immune Adherence Hemagglutination Test (IAHA)

(c) Radioimmunoassay

(d) Enzyme-linked Immunosorbent Test

(e) Hemagglutination Inhibition Tests

(f) Immuno Fluorescence Staining Techniques.

The traditional inexpensive simple complement fixation test is still widely used for routine diagnostic purposes. Although CF is sensitive enough for diagnosis of primary CMV infections,[33,34] it may not be sensitive enough for antibody detection among the seropositive individuals.[34] The antibody is almost always present in anybody who has had CMV infection previously, the level of the antibody may fall to very low levels.[34] Therefore, a very sensitive test in the laboratory sense (which can detect low levels of antibodies) is required to ascertain to a reasonable extent whether or not an individual has had CMV infection in the past.

Tests such as enzyme-linked immunosorbent assay and radioimmunoassay are highly sensitive but they have drawbacks such as non-specific reactions.[34]

Immune adherence hemagglutination appear to be a satisfactory alternative. This is a very sensitive test, 4 - 8 times more sensitive that CF, as shown by Castriciano.[35] Unfortunately not much has been done comparing the sensitivity of CF in cytomegalovirus infection. Miller et al.[36] showed clearly that for hepatitis A antibody detection IAHA is more sensitive than CF. It is well known that Treponema pallidum immobilization (TPI) test is a dependable highly sensitive and specific test for the serologic diagnosis of syphilis.[37] Miller et al.[38] compared the IAHA test with the TPI test for the diagnosis of syphilis and found a very high correlation of agreement between the two tests in terms of sensitivity and specificity. For example, TPI test was used to examine 41 sera. 21 were positive for syphilis and 20 were negative. When using IAHA, exactly the same 21 were positive as when using TPI, and those 20 that were negative on TPI were also negative on IAHA.[38] When the tests were applied to larger sample of serum (n = 69), 25 were known treated syphilitic serum and 44 reactive serum by TPI but not treated, the same trend was observed 20 of the 25 were positive on both tests, 2 were negative in each test, 3 were negative on TPI but positive on IAHA. In a 2 x 2 table, the data could be summarized as shown in Table 5.2. The sensitivity can be calculated for each of the tests thus:

TABLE 5.2

Whether TPI or IAHA	Disease (Syphilis) +	Disease (Syphilis) -	
Test Positive	21	0	
Test Negative	0	20	
	21	20	41

TABLE 5.3

	Disease (Syphilis)				Disease (Syphilis)		
Test (TPI)	Present	Absent	Total	Test (IAHA)	Present	Absent	Total
Positive	20	0		Positive	23	0	
Negative	5	0		Negative	2	0	
	25	0	25		25	0	25

for TPI sensitivity = $\frac{20}{25}$ = 80%

for IAHA sensitivity = $\frac{23}{25}$ = 92%

The fact that only known cases were tested in this case makes it impossible to calculate the specificity of the tests. If there were patients without syphilis tested, then the specificity could be calculated. From the above calculation, it is clear that IAHA is not less sensitive to detect disease than the well accepted TPI. The other 44 sera from untreated patients were also reactive on IAHA test.

Although it may not be entirely correct to assume that the same degree of sensitivity and specificity will be obtained when IAHA is used to detect CMV antibodies, the few data that are available do not lead one to think otherwise.[35,39] Part of the data of Dienstag et al.[39] is shown in Table 5.4.

IAHA is satisfactory in detecting antibody levels both in the acute and convalescent period. Another advantage of IAHA over CF is that whereas CF takes 12-20 hours to do, the IAHA can be completed in 5 hours.

However, experience with IAHA is not as extensive as with CF. Also, the end point in reading the test is not as definite as CF test. But with experience these difficulties can be overcome. There is also the possibility of non-specific reaction for either test no matter how small.

TABLE 5.4

ANTIBODY TO CMV BY IMMUNE ADHERENCE

Anti-CMV in Paired Sera of Multiply Transfused Patients[37]

Patient	Date	Anti-CMV Inverse Titer CF	IAHA
1	4 Weeks	2	64
	8 Weeks	128	2048
2	Pre	8	<8
	9 Weeks	65	256
3	Pre	32	256
	14 Weeks	128	1024
4	Pre	<4	<8
	24 Weeks	64	2048
5	4 Weeks	<2	<8
	11 Months	512	4096
6	Pre	32	256
	23 Weeks	128	1024
7	Pre	<2	<8
	19 Weeks	32	128
8	Pre	<2	<8
	17 Weeks	32	32
9	Pre	<2	<8
	22 Weeks	16	32
10	Pre	<2	16
	10 Weeks	64	512
11	2 Weeks	<2	32
	13 Weeks	64	256
12	Pre	<2	<8
	10 Weeks	64	256
13	Pre	<2	8
	20 Weeks	64	128
14	4 Weeks	<2	32
	10 Weeks	128	512
15	Pre	4	64
	24 Weeks	32	128
16	Pre	<2	<8
	17 Weeks	32	16
17	Pre	<2	8
	24 Weeks	8	64
18	Pre	<2	<8
	23 Weeks	8	512

Adapted from Dienstag et al.[37]

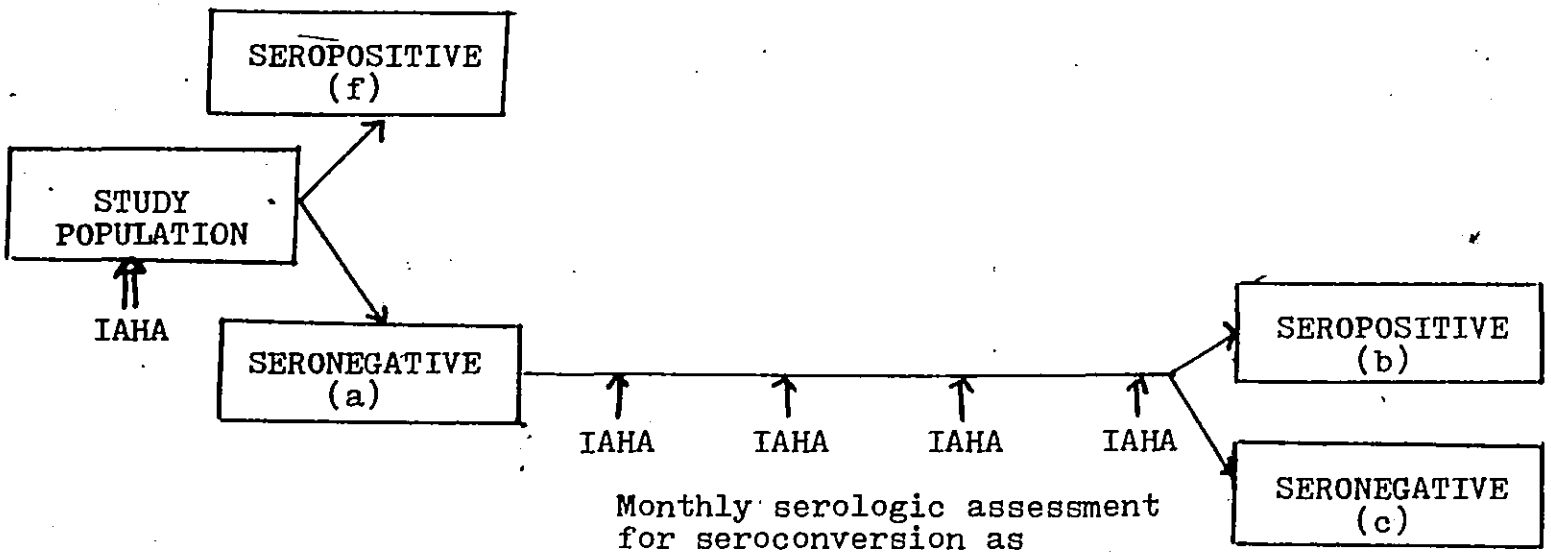
Therefore, for the incidence study the IAHA will be used to examine the sera.

Other problems with serological tests is the possibility of cross-reaction. Because CMV is one of the members of herpes virus group, there is the possibility of cross-reaction. Cross reaction implies that using CMV antigen, one may obtain a positive antibody response that does not belong to CMV but rather to any other members of herpes virus family eg. herpes simplex. The data of Castriciano[35] indicates that there is no cross-reaction when IAHA test is used to detect CMV antibody.

5.6 Design for the Study of Incidence

The total eligible population as defined in Chapter Two would be included. Their sera would be tested by IAHA test. This test will screen out those that have had previous exposure to CMV (seropositive). The individuals whose sera are negative would be assumed free from previous exposure to CMV. These seronegative individuals will form the cohort for incidence study. The proportion that are seropositive constitute the prevalence of CMV among that population. Monthly IAHA test will be done on the serum of these seronegative individuals in order to detect when they become seropositive if they do at all. The whole scheme will be represented as shown in Figure 5.1.

FIGURE 5.1



Overall Experimental Design to Study the

Inoidence of CMV

(i) $\frac{b + (W_x)(q_x/2) \times 1000}{a}$ / yr = Incidence rate (ii) $\frac{f}{f + a}$ = Prevalence

(W_x, q_x defined in section 5.1)

(i) $\frac{b + (W_x)(q_x/2) \times 1000}{a}$ / yr = Incidence rate

(W_x, q_x defined in section 5.1)

(ii) $\frac{f}{f + a}$ = Prevalence

The proportion that became seropositive within the specified time will contribute to the estimate of the incidence. For example: Suppose there are 100 seronegative patients followed monthly with serologic tests, and at the end of one year, 40 have become seroconverted. The incidence then = 40%.

In terms of how frequently the serologic tests should be done, certainly one can measure the antibody level at the beginning of the follow up of the seronegative cohort and measure the antibody levels again at the conclusion of the specified time. However, important information may be missed if such a method is adopted. By doing the tests frequently, if most of the early seroconverted ones have primary diagnosis of nephritis for example, this information will be missed if there is no early enough close look.

5.7 Principles of Immune Adherence Hemagglutination Test

This test is similar to the first half of complement fixation test. However, the indicator system is unsensitized erythrocyte. The end result is through agglutination,

brought about by the complement instead of the usual lysis of the complement fixation test. The reactions involved can be represented thus:

1. $Ag + Ab \longrightarrow Ab.Ab$

2. $Ag.Ab + C \longrightarrow Ag.Ab.C_{1423}$

3. $Ag.Ab.C + E \longrightarrow$ Hemagglutination

where Ag is antigen, Ab is antibody, C is complement and E is human group O erythrocyte. In the first step, the antigen reacts with the antibody, if any, to form antigen-antibody complex. Then, complement is added. Complement fixation proceeds to C_3 component and the human erythrocyte E (group O) is added as an indicator. The complex with the complement binds to C_{3b} receptors on the red blood cells resulting in agglutination rather than hemolysis. Usually ditrothreitol (DTT) is added to stabilize the agglutination. This agglutination is then observed macroscopically.

Again by serially diluting the serum, the test can be and usually is adapted to quantitative measurement of the antibody present in the serum in titers. The highest dilution at which agglutination occurs is the end point. Titer of < 1.8 is regarded as negative. The end point of IAHA is not as definite as CF test but with experience the results are rendered not only sensitive but also reproducible.

The test needs both positive and negative controls. Because there are many steps involved, the sources of errors are many and they are tests to be handled with care.

5.8 Materials for IAHA Test

(a) Microtiter Plates: The plates used may have different surface properties. This may result in non-specific reactions. Lennete[34] reported that the number on the lower right corner of the plate is the best way to check for bad plates. The same author indicated that coating the plates with buffer solution containing carrier protein will prevent errors due to low concentration of protein in the plate. The low concentration of protein may increase the non-specificity of the test unless buffer-protein is added.

(b) Reagents: The essential reagents for the IAHA consists of DTT, group O human red blood cells and veronal buffer saline containing 0.1% bovine serum albumin.

(c) Details of the Test: All serum samples will be labelled by name, hospital number and study number. The samples will be sent to the laboratory (virology) as described in section 4.5.2. On arrival at the virology laboratory, the technologist will receive them, enter the names and numbers into a log book (prepared for the study)

and the serum sample will be kept frozen until it is time to process the samples along with other samples. Weekly processing of samples will be adequate unless a study patient becomes sick. Under that circumstance, the serum will be processed the same day.

Technical details of performance of the test will be as laid down by Castriciano.[35] The results will be entered into the result book by the technologist who does the test.

5.9 Application of the IAHA Test to the Population For Incidence Study

When the criteria for admission into the study have been met, (advantage will be taken of the fact that these patients have left over recent serum from hepatitis surveillence tests), the sera from the patients in the study will be separated. The sera so separated form a retrolective sample and can be validly used to screen the patients for CMV antibodies by the IAHA test.

Applying the serologic test indicated above, the seropositive patients will be identified. They will no longer participate in the incidence study. The seronegative ones (already agreed to participate) will then be followed at monthly intervals for transplant and dialysis patients. Monthly intervals for these patients is chosen because in most reported cases, the bulk of post-transplant primary

CMV infection occurs within 6 months (Betts et al.)[13,79,86] Although the rate of seroconversion among dialysis patients may be slow, for comparative purposes both the transplant and dialysis patients must be tested at the same intervals. The study period will extend over 18 months for all patients. A single technologist will perform all serological procedures.

At the end of the study period, the number that become seropositive will be recorded as the numerator and total number at risk in the study as the denominator. This fraction expressed in percentage per year will form the incidence of CMV.

Layout of Tables for Analysis

5.10 (a) Table 5.5

1 = age

2 = sex

3 = primary diagnosis

4^a = duration of dialysis < 12 months

4^b = duration of dialysis ≥ 12 months

5^a = transfused

5^b = never transfused

6 = source of kidney transplanted

7 = date transplanted

8 = date last seronegative

9 = date first seropositive

TABLE 5.5

Pts	1	2	3	4^a	4^b	5^a	5^b	6	7	8	9	Initial IAHA	Highest IAHA	Change in Titre
1														
2														
3														
4														
5														
6														
7														
.														
.														
.														
n														

TABLE 5.6

5.10 (b)

P Δ = Primary diagnosis

Pts	P Δ	Age	Sex	5^a	4^b	Initial IAHA	Final IAHA
1							
2							
3							
4							
5							
.							
.							
.							
.							
n							

TABLE 5.7

5.10 (c)

Pts	Age	Source of Kidney	Initial IAHA	Final IAHA
1				
2				
3				
4				
5				
.				
.				
.				
.				
n				

5.10 (d) Calculations

Count how many became seroconverted in one year - say x. If the total seronegative population observed for one year = y, then incidence of CMV among this population = $\frac{x}{y}$ x 1000/year (assuming no deaths or withdrawals. See section 5.1 (b).

TABLE 5.8

Patient	(a) Age	(b) Sex	(c) 1^{o}	Duration Dialysis	Initial Titre	Final Titre	Change in Titre
1							
2							
3							
4							
5							
6							
7							
.							
.							
.							
n							

This data can then be analysed by multiple regression, using change in CMV antibody titre as dependent variable.

CHAPTER SIX

CELLULAR IMMUNITY AMONG RENAL TRANSPLANT AND DIALYSIS PATIENTS WITH CYTOMEGALOVIRUS INFECTION

6.1 Background

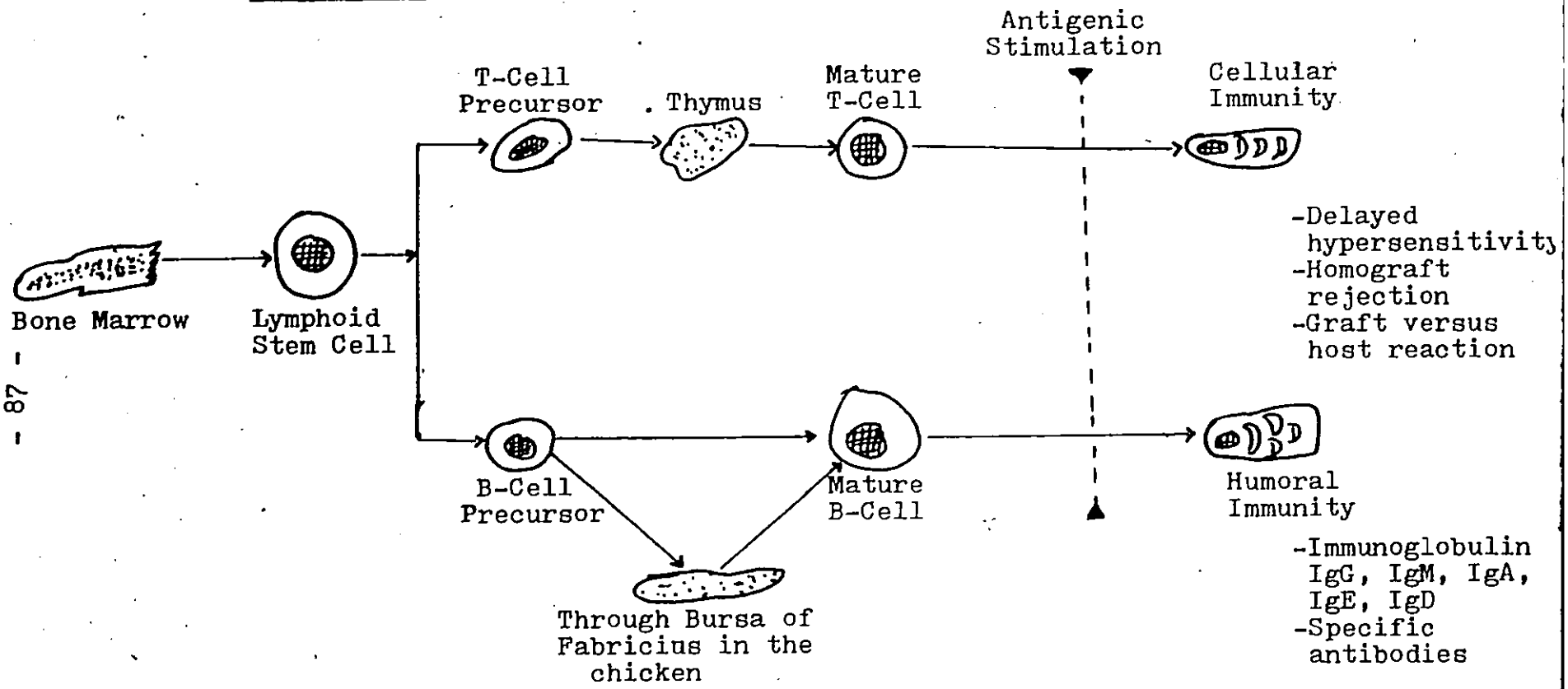
Practically every renal transplant patient is treated with immunosuppressive drugs such as azathioprine plus corticosteroid in an attempt to prevent allograft rejection. In some cases immunosuppressive therapy could result in severe and fatal virus infections.[10,12,16,42,61] Yet other renal transplant patients do not get the viral infection while on the same immunosuppressive therapy. The immunologic basis for such frequent viral (CMV) infection in renal transplant and dialysis patients is the subject of this chapter.

Immunity can be classified into two main types: (a) Humoral - which is antibody-dependent, and (b) Cellular - which is thymus dependent. Monitoring immunity in any patient requires the study of both humoral and cellular components. The findings can then be correlated with the course of the patient.

Both the humoral and cellular components of immunity originate from a common source - the bone marrow as illustrated in Figure 6.1 (page 87).

FIGURE 6.1

Development of Immunologic Systems - Cellular and Humoral



The diagram in Figure 6.1 is oversimplified. There are cross-links between the cellular and humoral immunities, one modulating the functions of the other. The conditions that are associated with either cellular or humoral immunity are indicated on the right-hand side in the diagram. The lymphoid stem cell can be said to be pluripotential in that it can give rise to either T-cells or B-cells. T-cells are lymphocytes that differentiate in the thymus (they are thymus-derived lymphocytes). They form about 80% of total circulating lymphocytes. Some of the T-cells maintain immunologic memory. They exhibit delayed (as opposed to immediate) hypersensitivity reactions. The T-cell subpopulation recognize and cooperate with B-cells (modulate immunoglobulin synthesis).[64] Some of the T-cells act as suppressor to B-cells and T-cells. Therefore, cellular immunity is under the influence of T-cells.

B-cells are lymphocytes that differentiate independently of the thymus. These lymphocytes mature at sites equivalent to the "bursa of fabricius" in birds. The anatomic site in humans where these lymphocytes develop is uncertain. These lymphocytes (B-cells) secrete immunoglobulins and specific antibodies as shown in Figure 6.1. The B-cells are 15 - 20% of the circulating lymphocytes. Humoral immunity (antibody mediated) is dependent on the activities of the B-cells.

The majority of individuals with deficiency of humoral immunity but intact cellular immunity do not have frequent serious viral infections; they have mostly bacteria infections. On the other hand, when an individual has deficiency of cellular immunity the capacity to control viral infections is compromised. He does not exhibit homograft rejection. He also fails to develop delayed hypersensitivity appropriately.[56] This is in keeping with the concept that the cell-mediated immunity is an important factor in host resistance to viral diseases. The works of Thurman et al.,[57] Glasgow et al.[56] and Fulginiti et al.[96] indicate that cell-mediated immune systems play an important role in combatting viral infections.

There are reports showing that among renal transplant patients on immunosuppressive drugs, CMV infection is very frequent, and in some cases severe.[12-17,19] Whether this frequent occurence of CMV infection among renal transplant patients is due to immunosuppressive treatment alone or has a multifactorial etiology is not yet resolved. The data of Dowling et al.[63] showed that the use of non-steroidal cytotoxic immunosuppressive drugs such as azathioprine in rheumatoid arthritis contribute significantly to the acquisition of measurable CMV antibodies by CF test. The data are shown in Table 6.1. Three of the 15 (20%) patients receiving either of the immunosuppressant agents developed

TABLE 6.1

CMV CF Antibodies in a Rheumatology Clinic Population According to Therapy[63]

	Reciprocal of CF Titer									
	<4	8	16	32	64	128	≥256	Total	1/GMT	Titer ≥1.8 (%)
Immunosuppressant Azathioprine or cyclophosphomide	5	0	0	1	0	5	3	14	30.5	64.3
Corticosteroid	18	1	4	3	4	0	1	31	6.5	41.9
No drugs	29	3	12	21	14	7	0	86	13.6	66.3
TOTAL	52	4	16	25	18	12	4	131	12.5	60.3

viruria whether or not they receive corticosteroids. It appears that the non-steroidal immunosuppressive drugs above predispose these patients to both detectable antibodies and viruria. Whether the viruria is of primary infection or is a reactivation is open to question.

Most of the study patients with CMV infection produce CF antibodies to the virus.[29,42,61,62] CF antibodies is a measure of humoral immunity. From the studies cited above and others, it appears that humoral immune response to CMV infection is not usually adversely affected by the immunosuppressive drugs. However, there are few reported exceptions to the above general statement.[42] In such cases, both the immunoglobulin and CF antibodies would be more than two standard deviations below the mean. Such patients usually have fatal outcome.[42] The data of Lopez et al.[42] illustrating these points is shown on Table 6.2.

There is evidence to suggest that CMV infection may depress cellular immunity. Howard et al.[54] have shown that in CMV infected mice, the survival of skin graft in the presence of a weak histocompatibility barrier is longer than in non-infected mice. This implies that the host failed to reject a "foreign" tissue. This functional impairment is T-cell dependent. In the same study, the investigator showed that CMV also inhibits the uptake of tritiated thymidine in response to phytohemagglutinin (PHA).

TABLE 6.2

Serum Immunoglobulins and CF Antibody Titres During Virus Infections In Immunosuppressed Renal Transplant Patients[42]

Patient	Serum	Immunoglobulins (mg/100 ml)*		CMV CF Antibody Titres
	IgG	IgM	IgA	Titres
R.S.	2240	450	370	≥1:4096
E.B.	1740	165	355	1:256
K.L.	1740	115	125	1:256
E.W.	1450	80	185	1:128
P.S.	690	40	215	<1:8**
R.H.	340	40	40	<1:8
G.L.	340	7	50	<1:8

* Normal values: IgG = 1158 ± 305 mg/100 ml; IgM = 99 ± 27; IgA = 200 ± 64

** CF 1:8 but 1 year later produced 1:16 after virus infection was detected by isolation

The degree of thymidine incorporation was significantly less in the CMV infected mice as compared to the control ($P < .001$). This inhibition of thymidine uptake lasted up to 26 days after the infection.

Definitions: (a) PHA is a lectin which is derived from the red kidney bean (Phaseolus vulgaris) which stimulates predominantly thymus dependent lymphocytes (T-cells). It is one of the non-specific mitogens. The mitogens activate T-cells in general. They can be used to assess T-cell function.

(b) Micro-PHA test is based on heparinized whole blood, and gives a quanitative estimate of the patients' thymus dependent (T-cells) function in the peripheral blood.

(c) Macro-PHA test is PHA stimulation test performed on purified and repeatedly washed lymphocytes to remove serum factors from the blood.

The data of Lopez et al.[42] indicated that while 61 renal transplant patients were on immunosuppressive agents, the mean response of their lymphocytes to micro-PHA stimulation was much greater than two standard deviations below the mean of normal ($P < .0001$). The same authors showed that patients on intensive anti-rejection therapy (usually azathioprine and steroids) always have a marked decrease in their micro-PHA responses. The micro-PHA

response reverts towards normal a few weeks after the discontinuation of the drugs. This suggests that the drugs may be the important factor in the lack of lymphocyte response to PHA. On the contrary, the macro-PHA test usually shows normal response in these patients. The response to macro-PHA stimulation (independent of viral infection) again suggests that the drug (serum factor) rather than CMV infection per se may be the main pathogenetic factor responsible for the depression of cellular immunity in these patients.

Also, using highly purified CMV antigen, Linnemann Jr. et al.[55] showed that lymphocytes from pre-transplant CMV seropositive renal patients exhibited blast transformation (stimulation index $\geq$ 3 defined in section 6.3.1). Whereas this lymphoblast transformation was lost in most of their patients within one month post-transplant (n = 15). This loss in cellular immunity was general (as demonstrated by PHA results in Table 6.3) but dramatic with CMV. There was some recovery in subsequent months particularly among those who had clinical CMV infection.

Other investigators have demonstrated similar blastogenic responses to CMV antigen in patients with CMV antibodies.[57,65,66]

TABLE 6.3

"Non-Specific" Mitogens Which Activate (Stimulate) Human Lymphocytes

Mitogens	Source	Relative Specificity
1. Phytohemagglutinin (PHA)	Phaseolus Vulgaris (Kidney bean)	T-Cells
2. Concanavalin A (Con A)	Canavalia ensiformis (Jack bean)	T-Cells (different subset from PHA)
3. Antilymphocyte globulin (ATG)	Heterologous Autisera	T-Cells
4. Pokewed mitogen (PWM)	Phytolacoa Americana	T and B-Cells (primarily B-Cells)

Adapted (modified) from Stites C.P. in Basic Clinical Immunology (1977) ed. by H.H. Fudenberg et al. [64]

6.2 Assessment of Cellular Immunity

Cellular immunity may be assessed by either delayed hypersensitivity skin tests or by lymphocyte activation. Apart from PHA, there are other non-specific mitogens which can activate human lymphocytes. They could be used to test for T-cell function. The problem with mitogens is that they are non-specific. Some of them are shown in Figure 6.2.

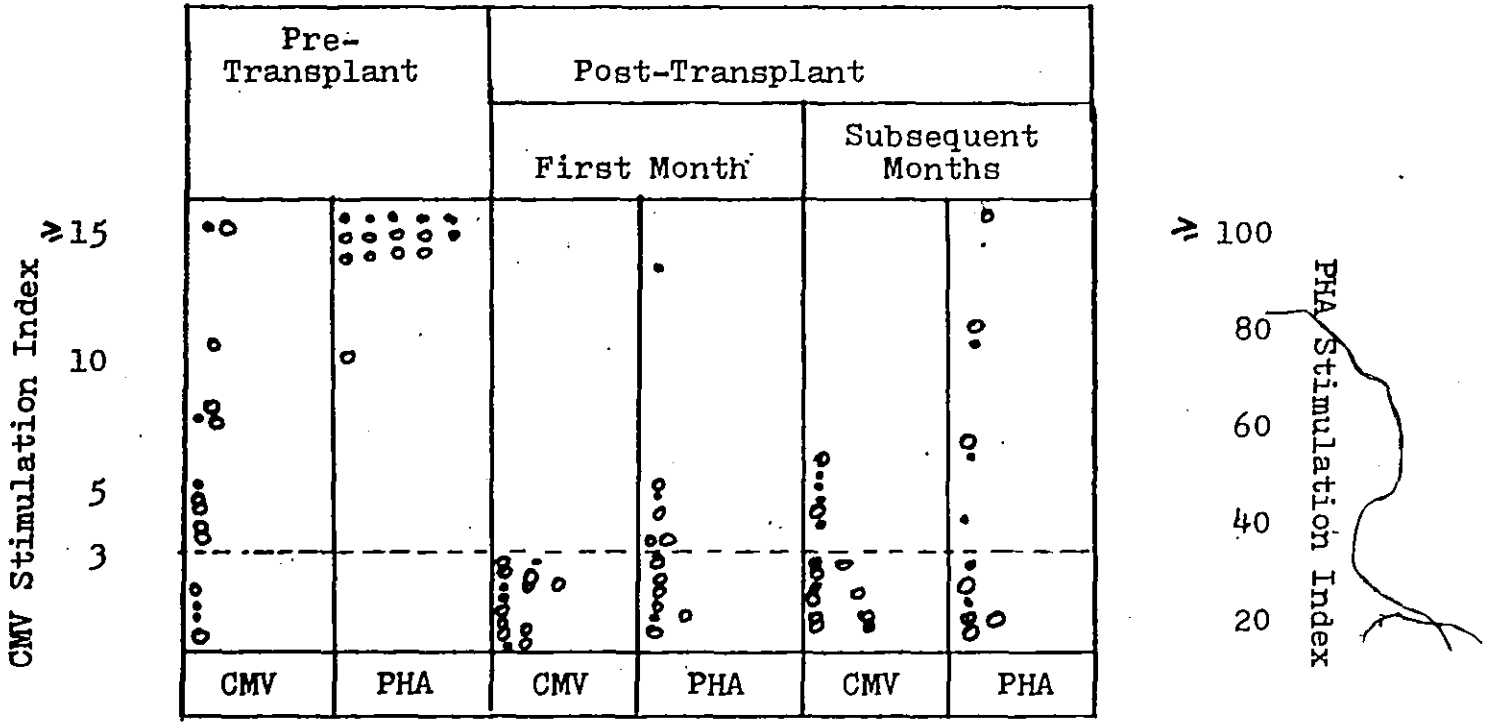
A more useful approach is the use of antigens rather than mitogens in assessing cellular immunity. The antigens stimulate fewer cells which are specifically sensitized to the antigen under investigation. Most antigens stimulate T-cells only. Commonly used antigens to assess human cellular immunity include Purified Protein Derivative (PPD), Candida, Streptokinase/Streptodornase, Vaccinia virus, etc.

Lymphocyte stimulation is an in vitro correlate of an in vivo process. When lymphocytes are stimulated as a result of an antigen interacting with the already sensitized lymphocytes, they are transformed into lymphoblasts. The process of transformation is blastogenesis.

Many biochemical changes take place following incubation of lymphocytes with mitogens or antigens. These changes result in increased synthesis of phospholipid, increased permeability to divalent cations, activation of

FIGURE 6.2

Lymphocyte Transformation in Autologous Serum to CMV Antigen and PHA in Renal Transplant Patients (Linnemann et al.)[55]



adenylate cyclase resulting in elevation of cyclic adenine monophosphate (cAMP). Shortly thereafter, synthesis of protein, RNA and finally DNA occurs.[64] Increase in DNA which results in cell division (multiplication) is in fact the biochemical basis for lymphocyte stimulation test.

This lymphocyte stimulation test can beused to assess cellular immunity in patients with immunodeficiency disorders, autoimmunity, cancer,infectious diseases.[64] It is a more reliable test of immunocompetence than merely counting types of lymphocytes (T- and B-cell assays) since it measures the functional capacity of lymphocytes to proliferate following antigenic challenge.

One very important source of variation in lymphocyte stimulation tests is the presence of suppressive factors in the serum. The response of lymphocytes may be suppressed by a variety of non-specific factors present in human serum. This humoral modulation is quite different from instrinsic suppression of cellular response. Their presence may be detected by careful history of the donor of serum and by doing a control test using washed lymphocytes from the donor and incubating it in normal serum. Stites D.P.[64] gave the list below as suppressive factors to avoid.

Lymphocyte Suppressive Factors in the Serum

Serum Proteins

Albumin (high concentration)

Specific antibodies to stimulating antigens

Immunoregulatory globulin

Alpha-1-acid glycoprotein

Pregnancy associated serum globulins

C-reactive protein

Serum alpha globulin of amyloid

Alpha globulins in cancer, chronic infection, inflammatory disease

Alpha feto protein

Low density lipoprotein

Antigen-antibody complexes

HLA antibodies

T-cell antibodies normal serum inhibitors

Hormones

1. Glycocorticoids
2. Androgens
3. Progesterone
4. Prostaglandins
5. Estrogens

Drugs:

1. Aspirin
2. Chloroquine
3. Cannabis
4. Quabain

Others

1. Interferon
2. Chalones
3. Cyclic nucleotides

6.3.1 Principles of Lymphocyte Culture

When a cell is taken out of the body (natural environment), it can be kept alive if the essential nutrients and a non-hostile environment can be provided. If lymphocytes are separated from blood, they would be kept alive if the above conditions are met. Therefore, it is important to provide appropriate nutrients and environment (biochemical, thermal, etc.) for lymphocytes in culture. Usually the culture medium meet the nutritional and biochemical requirements. The thermal requirement is adjusted externally.

When mitogens or antigens are added to the lymphocytes and incubated then the series of biochemical changes described in section 7.2 takes place. To facilitate this, the culture is kept in 5% CO_2 environment. For mitogens

incubation for 72 hours is appropriate. For antigens 5 - 7 days are required (details discussed below). By the end of the incubation time, the lymphocytes have been stimulated, DNA synthesis as well as division have taken place. Suppose there were 1000 lymphocytes to start with and by the end of the test there could have been 5000. The lymphocyte stimulation index is 5000/1000 = 5. This is equivalent to saying:

$$\text{Stimulation Index (S.I.)} = \frac{\text{Count at end of test}}{\text{Count at beginning of test}}$$

Originally, morphologic assessment by counting the lymphoblasts was the method used. This method is no longer used because of extreme variability and subjectivity of the results. Since the rate of DNA synthesis determines the degree of blastogenesis (lymphoblast formation), measuring the DNA synthesis is a more accurate way of assessing the stimulation index than the traditional lymphocyte count.

Advantage is taken of the fact that DNA can be radio-labelled. Pulse-labelling the culture with tritiated thymidine results in the DNA bearing lymphoblasts to take on some of the radio-active substance. The amount of radioactive material taken up can then be measured by autoradiography, grain count or scintillation counts in a liquid scintillation spectrophotometer. The later method is preferred in most clinical immunology laboratories.

6.3.2 Sources of Variation in Lymphocyte Culture

Lymphocyte stimulation test, using specific antigen, has many sources of variation. The concentration of the cell (lymphocytes), the strength of the antigen, contamination of the cells with non-lymphoid cells, presence of lymphocyte suppressive factors in the serum, duration of the culture as well as dose-response kinetics of the culture system are few of the many sources of variation. The technics of harvesting the cells as well as scintillation counting problems (discussed below) may adversely affect the results.

For this study, the concentration of CMV antigen is very important. CMV is a virus for which antigen preparation can be difficult. The right pH is essential. Almost 100% cytopathic effect is required for antigen preparation.[65]

Culture time and dose response kientics are very important. Such kinetic curves are shown in Figures 6.3 and 6.4 for both the PHA and antigen.

As shown in Figures 6.3 and 6.4, the optimum concentration of the PHA for maximum response (100 - 200 μg/ml) is different from that of most antigens (15 μg/ml). The dose-response curves observed may indicate the fact that when the concentration of the antigen is too weak the stimulation is minimal. As the concentration increases, the stimulation also increases until maximum stimulation

FIGURE 6.3

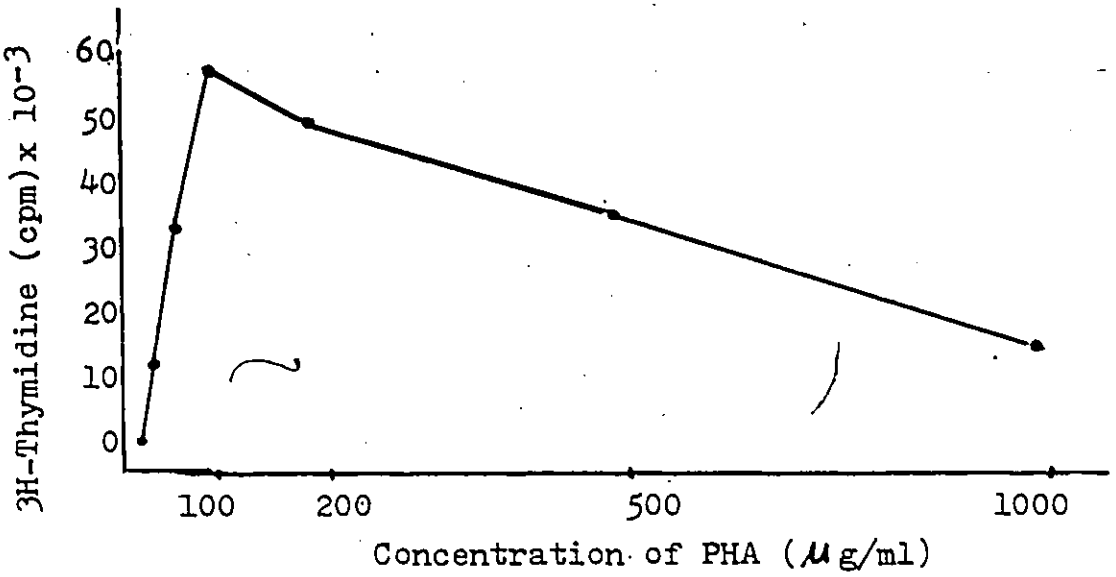
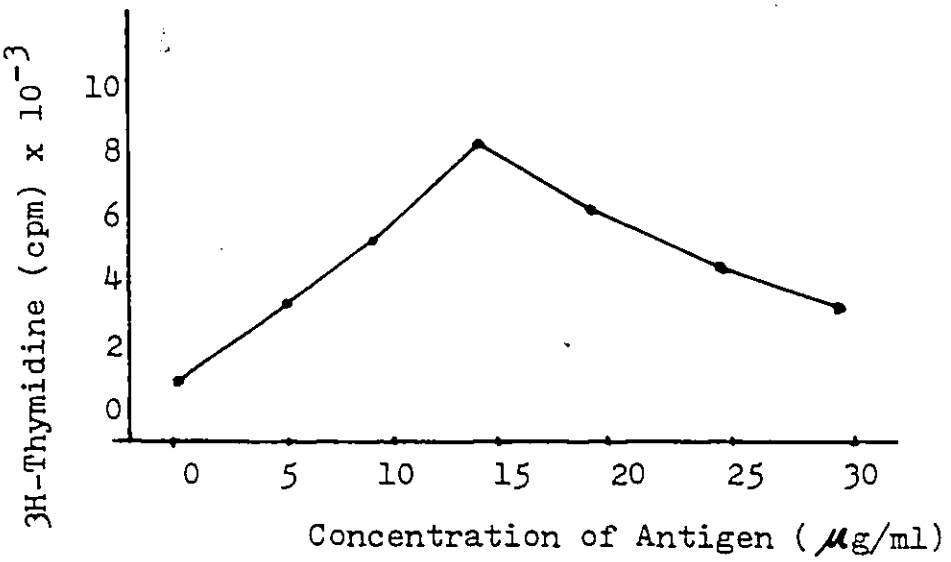


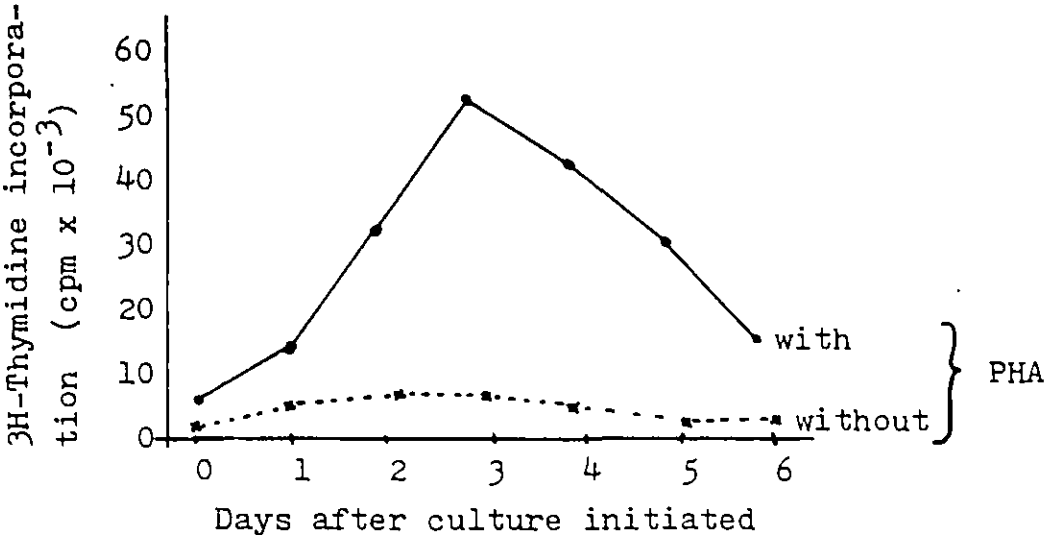
FIGURE 6.4



point is reached. Beyond that point, no further increase in stimulation takes place possibly because the concentration of the antigen may be toxic to the cells.

Similarly, the duration of incubation is important. Figures 6.5 and 6.6 below illustrate the type of time-response curve that one may obtain for PHA and for specific antigen.

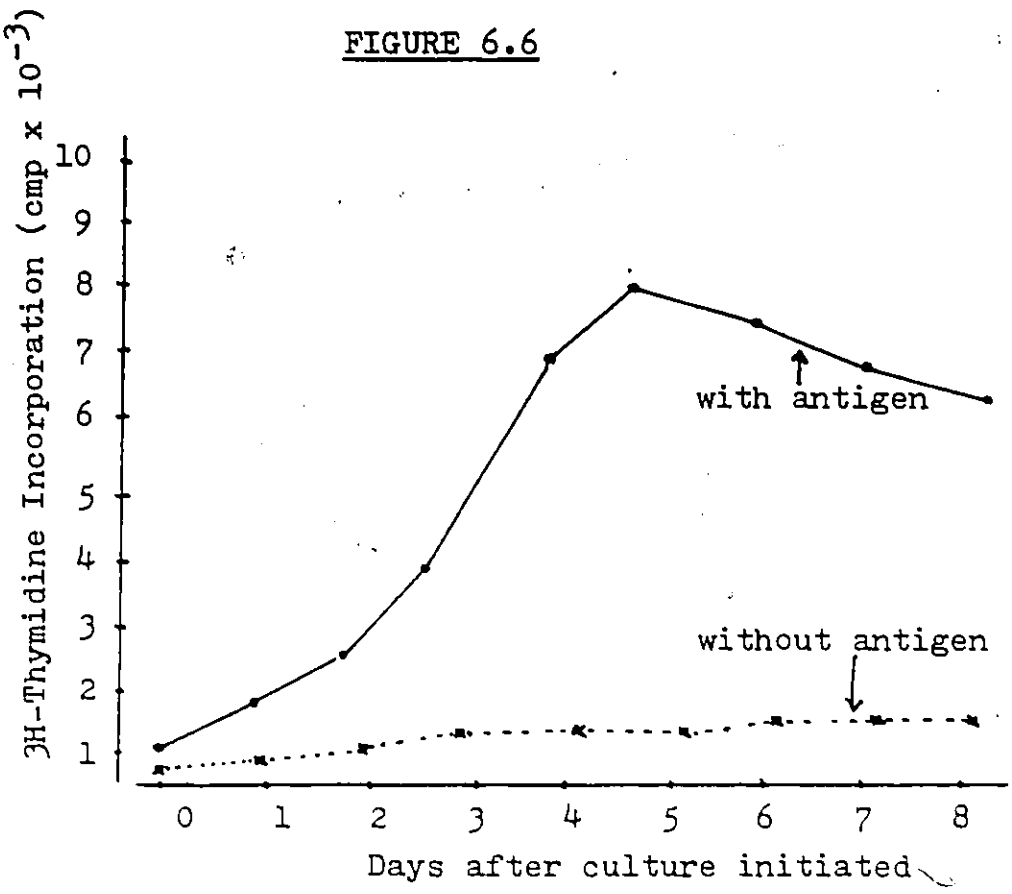
FIGURE 6.5



This diagram shows that the optimum duration for the incubation of lymphocytes for PHA stimulation is about 72 hours (3 days). Beyond 3 days, because the cells begin to die off, the thymidine incorporated becomes progressively less.

Similar trends are observed when lymphocytes are incubated with specific antigen as shown diagrametically in Figure 6.6.

FIGURE 6.6



As shown in Figure 6.6, the optimum duration of incubation is 5 - 7 days. Specifically with CMV, the data of Waner et al.[65] supports the duration of culture to be 5 - 7 days. Therefore, to harvest the cells in less than 5 days or more than 7 days of culture may affect the stimulation index.

6.3.3 Tracing Lymphocytes by Radioactive Labelling

Lymphocytes may be trace labelled for the study of physiological migration of the cells from blood into the spleen, lymph nodes; and for the identification of different cell populations such as long-lived and short-lived

lymphocytes. Lymphocytes may also be trace labelled for measurement of increase in the rate of cell proliferation in response to mitogens or antigen by incubating lymphocytes in a medium containing tritiated thymidine (3H-thymidine). For the present study, the lymphocytes would be trace labelled with tritiated thymidine for assessment of lymphocyte proliferation as a response to CMV antigen challenge.

It is important to remember that there are different radioactive substances that can be used for trace labelling lymphocytes. The choice of a particular radioactive material depends on the objective of the test. The choice of the radioactive substance depends on whether the labelled lymphocytes are to be detected by autoradiography, by counting whole organs or by scintillation count in a liquid medium.[67] The Table 6.4 shows selected methods for trace labelling lymphocytes.

There are difficulties that must be appreciated in the interpretation of results of trace labelling lymphocytes. Dead cells may release radioactive trace which other cells may re-utilize. The labelled cell may be damaged by intranuclear radiation. It has been advised that in general the concentration of radioactive thymidine should be kept to a minimum.[67] When necessary re-utilization can be reduced by giving an excess of non-radioactive thymidine.

TABLE 6.4

Summary of Selected Methods for Trace Labelling Lymphocytes[67]

Isotope	Compound	Method of Labelling	Dosage	Distribution of Label Among Cells
^{3}H	Uridine-5-^{3}H	In vitro	5 μCi/ml Cells at 5 x 10^{7}/ml	Thymus derived small lymphocytes. All large lymphocytes
^{14}C	Uridine^{14}C (U)	In vitro	1-2 μCi Cells 5 x 10^{7}	As above
^{3}H	Thymidine 6^{3}-H	In vitro	0.1 μCi	Exclusively large lymphocytes (20-100%), depending on time of incubation
^{14}C	Thymidine^{14}C (U)	In vivo. Daily injections for 2 days	0.1 μCi/gm/day	Some long lived small lymphocytes
^{51}Cr	Sodium Chromate	In vitro	4-50 μCi/ml cell x 10^{8}	98-99% of all lymphocytes Uptake positively correlate with cell size.

Another factor to be considered is the presence in the serum of antibody directed against stimulating antigen. This may result in antigen-antibody complex formation which in turn may block or non-specifically stimulate lymphocytes.

6.3.4 Dye Exclusion Test

One of the factors that may result in low stimulation index is cell death. The cell death could be a result of any of the factors discussed early such as concentration of the antigen. The dye exclusion test is appropriate in determining the proportion of cells that are living at different times. The test utilizes trypan dye. A sample of the cells is harvested and 10% solution of the dye is added. Then look at the cell under the microscope. The dead cells will take up dye and will present a blue coloration whereas the living cells do not take up dye. The percentage of the living cell (that do not take up dye) can be calculated. The test may be repeated serially. To ascertain cell survival, it is useful to perform the test each time lymphocyte culture is set up.

A simple protocol for this test can be set up thus: Do viability count by mixing 1 drop of cell suspension and 1 drop of 0.4% Trypan Blue Stain. Immediately drop mixture into a counting chamber and coverslip. Count number of cells stained and total number of cells in the field.

Calculate % viability by:

$$\frac{\text{No. cells not stained}}{\text{Total number of cells}} \times 100 = \% \text{ viable cells.}$$

6.3.5 Lymphocyte Concentration and % Recovery

Another important source of variation in lymphocyte stimulation is the number of lymphocytes in the test solution (i.e. concentration of cells). It is important to separate the lymphocytes, concentrate the separated lymphocytes and actually count them. The concentration can then be adjusted to the desired level. This is usually about 3×10^6/ml.[87] With this concentration, it is assumed that there will be enough T-cells for blastogenesis. It is also useful to know the % of the lymphocytes recovered as a result of lymphocyte separation. If the percentage recovered is low (say 60%) then more blood will be required to obtain a final concentration of 3×10^6/ml cells. By using the figure from total and differential white blood cell count, the number of cells that should have been recovered is known. Also from the post-lymphocyte separation cell count, the actual number of lymphocytes recovered is known. Therefore, percentage recovery =

$$\frac{\text{No. of cells recovered}}{\text{No. of cells should have recovered}} \times 100$$

$$= \frac{\text{No. of cells recovered}}{\text{No. of cells that should have been recovered}} \times 100\%$$

By proper adjustment of the concentration of lymphocytes before cell culture, this source of variation in the result of the experiment can be minimized.

6.4 Principles of Radioactive (Scintillation) Counting

An important step in lymphocyte stimulation test is the radioactive scintillation counting. The counts per minute is the amount of radioactive uptake by the lymphocytes. This is a measure of the lymphocyte stimulation (blastogenesis). This step of radioactive counting constitutes another major source of variation in the overall result. It is therefore important to understand the principles of scintillation counting and the source of error of the counting. Such errors may then be minimized by taking essential precautions.

6.4.1 Definitions

(a) Scintillator: A scintillator is a substance which emits a weak light flash of short duration whenever it is struck by an ionizing particle. The intensity of the scintillation depends on the energy of the particle dissipated in the scintillator.[67]

(b) Organic liquid scintillator consists of one or more fluorescent aromatic solutes in an aromatic solvent,

to which other materials may be added to incorporate the radioactive specimen.[67]

(c) Photomultipliers: These are tubes which are sensitive to weak light signals, and which convert them into amplified electrical pulses.

Radioactivity

When the radioactive substance has been well dissolved and the particles of the substance is carried by a scintillator, these particles would emit light energy. For counting purposes, the liquid is kept in a vial (glass or plastic). The vials are put between two devices sensing light being emitted by the scintillation process. These sensors are the photomultiplier tubes.

When photons of light strike the photocathode with a light, sensitive surface, electrons are drawn from photocathode surface (photoelectrons), attracted towards several anodes. Several electrons are released from each electron striking the anode surface. By repeating this process from anode to anode with increasing number of electrons at each surface, a large number of electrons arrive at the final anode of the multiplier tube. The same process occurs at the second photomultiplier tube independently except when they both receive photons originating from the same scintillation flask in the scintillation vial between them. The anode collects the electrons from the last dynode

(electrons which generate secondary electrons) and produce a voltage - pulse proportional to the electrons collected.[61] The voltage pulse from the photomultiplier is proportional to the energy of the beta-particles emitted by the scintillator.

The design of the counter is complicated. This helps to minimize some of the numerous sources of error in radioactive counting. Figure 6.7 is a very simplified scheme of Beckman beta-counter. Because photocathode will release electrons when the temperature is high ("Joule-Thompson noise" or "background noise") the coincidence gate (or coin gate) are set to offset such extraneous noises. The pulse height is analysed to decide whether it is a real radioactive scintillation or not and the resultant pulses are stored in the bin (scaler). The stored pulses go through electronic calculator (not shown) where the pulse-height (intensity) are converted into counts. The counter is programmed to a typewriter which produces the printout of the counts.

6.4.2 Sources of Error in the Printout Counts

The sources of error in the counting are many because the process of counting involves many steps.

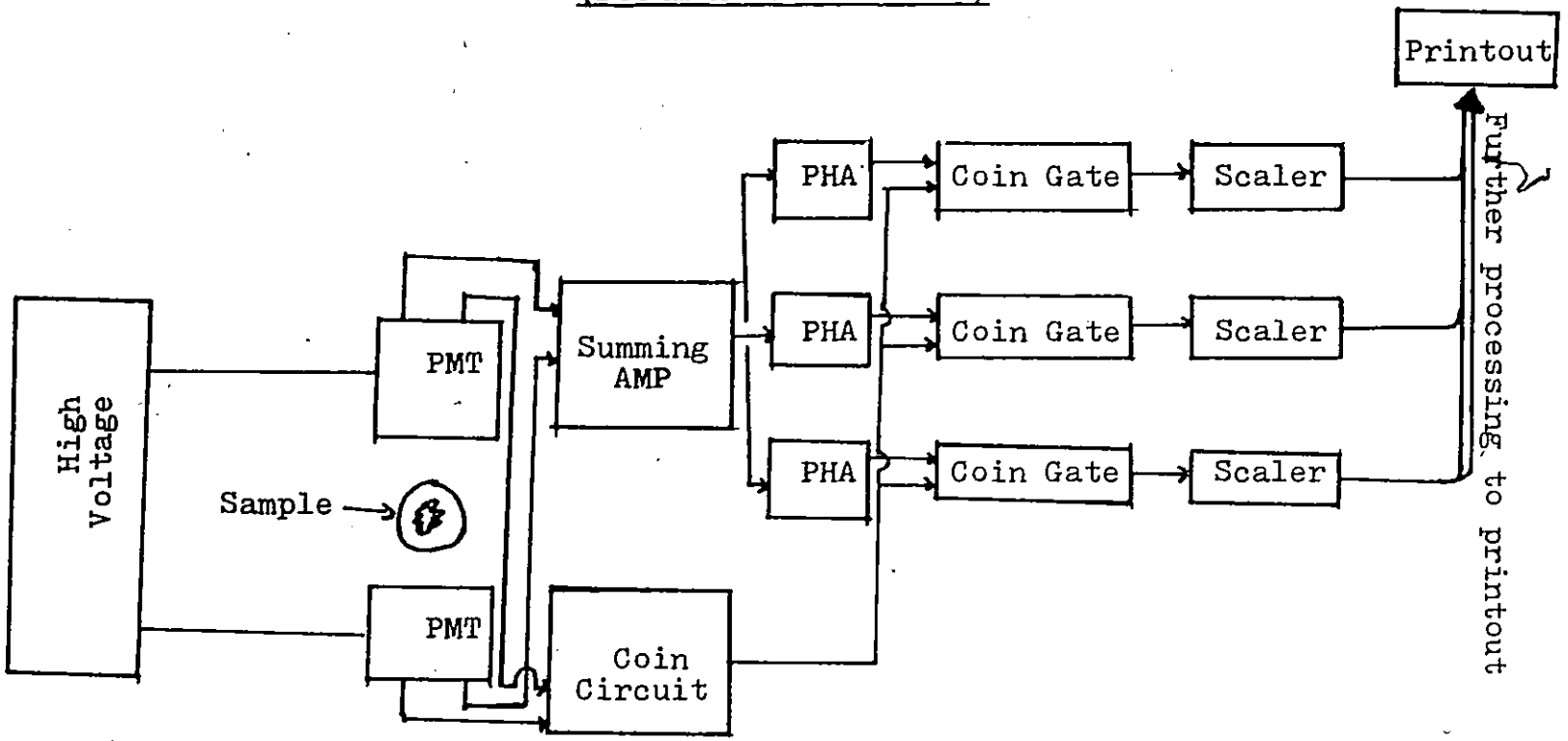
(a) Background noise can be dealt with by setting the counter coincidence gate appropriately.

(b) The most important practical source of error is quenching. Quenching is defined as any process which

FIGURE 6.7

General Scheme of L.S. Counter with Log Amplification

(Beckman Beta Counter)[68]



Key: PMT = Photomultiplier; PHA = Pulse height analysis; Scaler = Storage bin (before transferred out for print)

reduces the photon output from the counting system.[68] It will ultimately reduce the count rate. There are four main types of quench: (i) chemical, (ii) colour, (iii) dilution and (iv) absorption.

(i) The chemical quench does so by either competing with the fluors for energy transfer (resulting in less energy output by the fluors) or by chemically interacting with the fluors.

(ii) Colour quench results where the colour of the solution in the vial can in fact absorb the photons before they can be detected by photomultiplier.

(iii) In dilution quench, there is insufficient amount of fluor molecules in the solution in the vial. With appropriate concentration of fluor, this problem can be resolved.

(iv) Beta absorption quench: If the radioactive sample is not well dispersed in the scintillator, physical contact of the molecules of the radioactive substance with the scintillator will diminish. Beta particles which pass through non-scintillation medium will lose some of its energy and it will be presented as a weak beta energy. The final count in such a case will be low.

There are appropriate standardizations and calibrations to deal with quench problems. It is however very important to recognize it as quench when it occurs.

It is also important to remember that liquid scintillation counter is a passive observer, reporting how many flashes of light is coming from the vial in a given period of time. Therefore, every effort must be made to minimize impurities getting into the vials.

Decay: This refers to the natural loss of radioactivity from a substance over a time period. This is usually important where the substance loses radioactivity very quickly. In such cases the count should be made as soon as possible. In this study, tritiated thymidine would be used. The half-life of the radioactivity in tritiated thymidine is 12.3 years. Therefore, it does not make any difference whether the count is done the second day after pulsing or months later.

Radiation Protection: It is realised that radioactive substances are very hazardous to health and all the standard precautions laid down by the radiation authorities would be strictly adhered to when handling the specimens.

6.5 Overall Experimental Design to Study Cellular Immunity

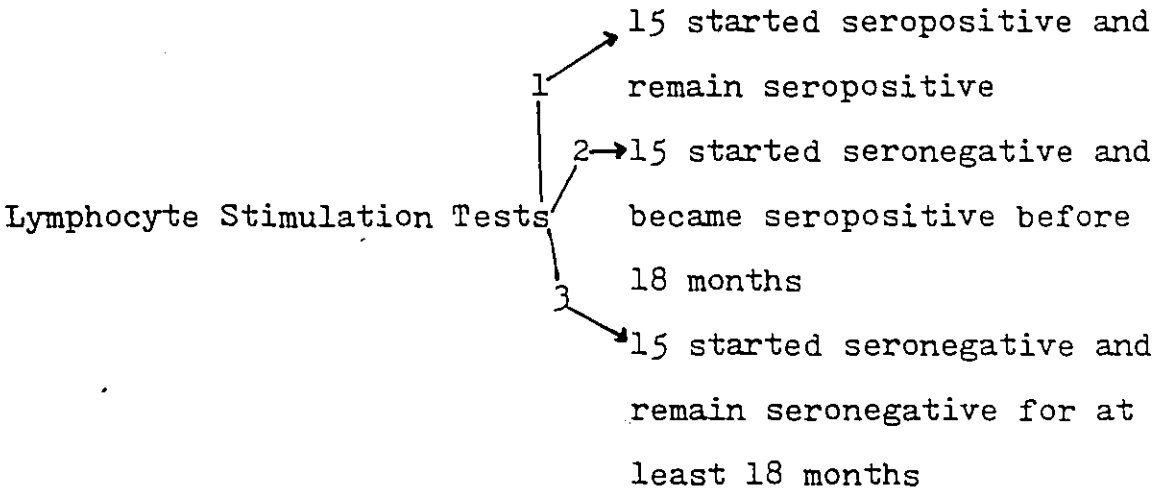
6.5.1 Study Patients and Their Characteristics

The study patients would consist of those who have freely agreed to participate in the study and who have given the free informed consent as described in section 4.4.3. The initial patient population in the study would have

their sera examined for CMV antibodies by IAHA. The population would have been divided into (a) those who have CMV antibodies (seropositive) and (b) those who do not have antibodies (seronegative).

From the seropositive group, a sample of 15 would be selected by standardized randomization for the lymphocyte stimulation tests (Group 1). Of the seronegative group, 75 individuals would be selected by standardized randomization for lymphocyte stimulation tests. Some of these will seroconvert (Group 2) and some will remain seronegative (Group 3).

These groups and the study manuevres would be represented as shown below.



The duration of the study would be 18 months for each patient. If a patient becomes seroconverted less than 6 months before the conclusion of the 18 months study period, such a patient would be studied beyond 18 months so that

every seroconverted patient would have at least six months of cellular immunity study post-seroconversion. The general scheme of the study would be as presented in Figure 6.8.

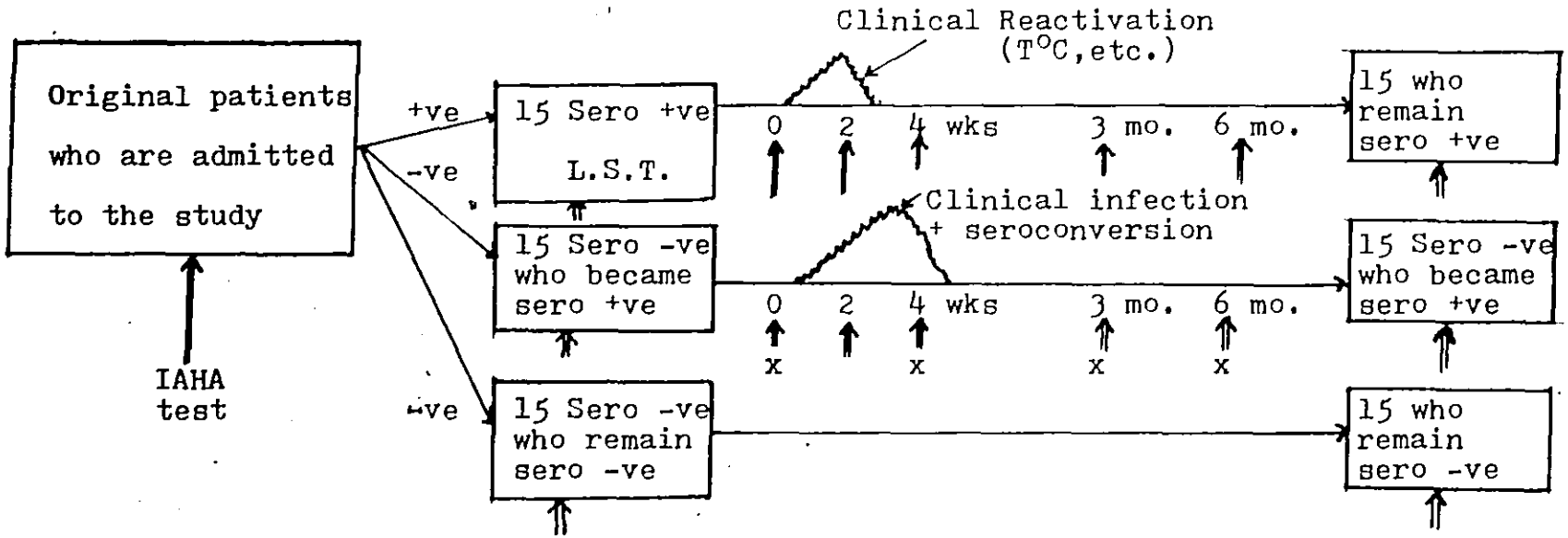
This study of cellular immunity would run concurrently with the incidence study as well as the morbidity/mortality study.

6.5.2 General Description of the Lymphocyte Stimulation Tests

The lymphocyte stimulation test is done in triplicate using both CMV antigen and non-specific PHA as stimulents. The control antigen is uninfected culture cells. The antigens as well as the PHA are diluted with a diluting medium RPM 1640. Blood is drawn into heparinized tubes from the patients (10 ml). The blood is diluted (1:1) in Hanks solution and the lymphocytes are separated by the use of sterile Ficoll-Hypaque gradient. Adjust the fluid volume to obtain 3×10^6 cells/ml. Viability study is then done on the cells using 0.4% Trypan dye test as described in section 6.3.5. This ensures not only the number of the lymphocytes but also the viability of the cells.

This cell solution is then added to the culture medium in the ratio of 0.5 ml of cells to 4.5 ml of medium. The mixture of the lymphocytes and the incubation medium is now incubated at 37° with 5% CO_2 for 7 days. Following the incubation, the mixture is then pulsed with tritiated thymidine 20 of 10 Ci/ml. This is then reincubated for 5 hours. It is then terminated by freezing ($20^\circ C$).

FIGURE 6.8



⇑ = Lymphocyte stimulation tests

x = Measurement of immunoglobulins

Overall Experimental Design to Study Cellular Immunology

Study Patients and Their Characteristics

The frozen culture plates contents are thawed, the contents are washed into scintillation vials. The vials are counted in a beta-counter for radioactivity. The mean of the three counts (triplicate) is then recorded as the average counts per minute. The mean counts among the seropositive asymptomatic patients can be compared with (a) when they are symptomatic, (b) the seronegatives, (c) seronegative that become seropositive, and (d) comparison can also be made about the duration of cellular immuno-depression following acute CMV infection in Groups 1 and 2. Dummy tables are presented for analysis of the data (Table 6.5).

TABLE 6.5

Results of Radioactive Counting: Both Seropositive and Seronegative Patients

Patients	Initial Study Mean	Mean Individual at Reactivation or Seroconversion	Mean Individual at Conclusion of study
1			
2			
3			
.			
.			
25			
	Mean (group) X_1	Mean (group) Y_1	Mean (group) at end of study Z_1
1			
2			
3			
.			
.			
25			
	Mean (group) X_2	Mean (group) Y_2	Mean (group) Z_2
1			
.			
.			
25			
	Mean (group) X_3	Y_3 Mean (group) not applicable	Mean (group) Z_3

Using appropriate statistics (discussed in Chapter Eight) the groups X_1 can be compared with X_2, X_3. Also compare Y_1 with Y_2. Finally Z_1 can be compared with Z_2, Z_3.

CHAPTER SEVEN

STUDIES ON THE MORBIDITY AND MORTALITY ATTRIBUTABLE TO CMV

7.1 Introduction

Since 1964, there has been an ever increasing number of reports confirming the earlier observations that CMV infection can inflict a significant morbidity on patients with renal transplant.[4,5,9-19] These reports emphasize the fact that the consequence of CMV infection has a wide spectrum even in patients on immunosuppressant agents, ranging from low grade pyrexia to fulminent pneumonitis, hepatitis, encephalitis, hemorrhagic pancreatitis and death. In 1964, Hill et al.[46] described terminal pulmonary infections in 26 of 32 patients dying post-transplant; 15 of these were associated with CMV. In 4 of the 15, only CMV could be detected at autopsy. These 4 had demonstrable inclusion bodies. In 1965, Hedley-Whyte and Craighead[12] reported a case of generalized cytomegatic inclusion disease following renal transplantation. Other investigators[10,11,13-19,60,88] confirmed the findings that pulmonary involvement is a very frequent occurrence in CMV infection. Usually, it presents with pneumonitis characterized by cough, tachypnoea, and rales in the chest. Fever may be the only symptom. Typical x-ray changes are characterized by 2 - 4 mm nodular densities

scattered throughout both lungs.[10] While this radiologic finding appears specific for pulmonary CMV infection, it is not sensitive as it was present in only 27% of cases with this pathologic finding.[10] These patients with CMV related pneumonitis patients can become extremely sick, hypoxic[12,15,16] and hypotensive[15] with resultant respiratory failure[15] and death. About 50% of renal transplant patients with CMV infection have pulmonary involvement.[16,18] At autopsy, the characteristic inclusion cell is found. Clinically the pulmonary signs and symptoms can be summarized thus: breathlessness, cough, increased respiratory rates and rales bilaterally. Radiologically there is bilateral peri-hilar distinct 2 - 4 mm nodular lesions in the lungs. When there was pulmonary involvement, all previous patients had viraemia.[17]

Pyrexia is another feature of active CMV infection among renal transplants and dialysis patients. The temperature may reach 103°F and may last 3 - 4 weeks.[17] Fever in association with arthralgia occurs in about 77% (n = 26) of cases.[17] The arthralgia has been described as characterized by sudden onset of stabbing pain involving the knees, hips, ankles, sacroiliac joints and wrists in that order of frequency.[17] In the study reported by Fiala, 19 of 20 with the combination of fever and arthralgia among renal transplant patients had active CMV infection.[17]

Swansirikul et al.[68] reported that 13 of 18 post-renal transplant patients who developed primary CMV infections had temporally related two or more of the following symptoms: fever, leukopenia, atypical lymphocytes, lymphocytosis, hypatosplenomegaly, myalgia, arthralgia and pneumonitis. On the other hand, only one of eight reactivated infections had similar combination of symptoms. 15 of their 18 primary CMV infections were symptomatic. Only one of ten secondary (reactivated) infections was symptomatic. The frequency of signs and symptoms among patients reported by Swansirikul et al. is as shown in Table 7.1.

In this study, at the time of seroconversion, 78% had fever, 67% had leukopenia and 67% had atypical lymphocytes. This data demonstrated a difference in the frequency and severity of morbid factors (listed under manifestations) among patients with primary CMV infection as compared with those who had reactivated infections. This is contrary to the findings of Fiala et al.[17] who could not demonstrate any difference between the morbidity experienced by those who had primary as compared to secondary CMV infections. The data of Swansirikul et al.[68] is a partly retrospective study. It is possible that documentation of signs and symptoms were not accurately and completely recorded for all the patients. Therefore,

TABLE 7.1

Frequency of Selected, Unexplained Signs and Symptoms

In Patients With and Without CMV Infection[68]

Manifestation	No Infection		Primary Infection				Secondary Infection			
					Temporally Related				Temporally Related	
	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
Fever	1	(5)	14	(78)	14	(78)	4	(40)	2	(20)
Respiratory	7	(37)	7	(39)	4	(22)	5	(50)	1	(10)
Arthralgia	0	(0)	1	(6)	1	(6)	0	(0)	0	(0)
Myalgia	0	(0)	4	(22)	3	(17)	0	(0)	0	(0)
Hepatomegaly	1	(5)	4	(22)	4	(22)	0	(0)	0	(0)
Splenomegaly	0	(0)	3	(19)	3	(17)	0	(0)	0	(0)
Leukopenia	2	(12)	13	(72)	12	(67)	2	(20)	1	(10)
Lymphocytosis	9	(0)	2	(11)	2	(11)	1	(10)	1	(10)
Atypical lymphocytes	1	(5)	12	(67)	12	(67)	3	(30)	1	(10)
Abnormal Liver Function Test	3/16	(19)	3/13	(23)	3/13	(23)	0	(8)	0	(8)

the results the authors presented must be interpreted with caution.

Active cytomegalovirus infection has been associated with hepatitis among renal transplant patients.[29,69,70] Also, Luby et al.[27] described 20 of 44 renal patients that developed hepatitis in association with CMV infection. Other investigators cited instances of hepatitis in association with this virus infection.[13,16,17] In few instances, the bilirubin rose above 3 mg% along with elevated liver enzymes in the serum. The extent to which these findings can be said to be morbid has not been investigated.

Rejection of allograft has been associated with CMV infection.[17,47] The data of Lopez et al.[47] strongly incriminated CMV as a causal factor. The authors found rejection episodes in 19 of 29 CMV infected renal transplant patients. Therefore, in the study of morbidity experienced by CMV infected renal transplant patients, rejection episodes should be considered an important factor.

Encephalitis has been reported in association with CMV infection but infrequently among renal transplant patients.[16] Other clinical features of CMV infections include hypotension, heartburn and hemolytic process have been described.[69,71]

7.2 Definitions, Explanations of Morbid and Co-Morbid Factors

7.2.1 (i) Morbid state is ill-health or sickness or disease state. The severity of morbidity may vary from mild feeling of unwell to critically sick situation.

(ii) Mortality means death.

(iii) Pyrexia (fever) is defined for the purpose of this study as temperature of $\geq 38^{\circ}C$ orally lasting at least two days in any given seven days.

(iv) Pneumonitis is defined as breathlessness and increased respiratory rate (≥ 30/min.) plus rales in chest plus 2 - 4 mm nodular densities radiologically scattered in lungs bilaterally. Combination of radiologic findings (as above) plus any one of the signs or symptoms listed confirms the diagnosis of penumonitis for the purpose of this study.

(v) Hepatic dysfunction is defined as a situation in which the serum liver enzymes as well as bilirubin are elevated. SGOT greater than 65 I.U. is elevated. Bilirubin greater than 1.5 mg% is elevated.

(vi) Leukopenia is ≤ 4500 WBC/mm^3; lymphocytosis is lymphocyte count of at least 1000/mm^3. It is also 40% of total WBC. Increase in atypical lymphocyte count is defined as $> 2\%$ of total WBC count.

(vii) Allograft rejection is defined as a deterioration in renal function with resultant sustained increase in serum creatinine of at least 0.2 mg/100 ml with a concomitant fall in creatinine clearance.

(viii) Arthralgia is defined as pain in any joint lasting up to 12 hours or lasting up to 3 hours but recurrent within 24 - 48 hours. The pain may be mild, moderate or severe. Mild pain is recognized by the patient but it does not limit him/her in any way. Moderate pain is recognized and activity limiting but does not completely incapacitate the patient. Severe pain is severe arthralgia which completely incapacitates the patient during part of or the whole of its duration. There is no redness to the joint and it is not warmer than the corresponding other joint.

(ix) Bed disability day is defined as a day when patient stays in bed more than half of the day.

(x) Restricted activity day is defined as a day when activity is restricted to half or less than the normal good day.

7.2.2 Co-Morbidity

When there is a single morbid factor operative, the resulting disease can be linked with the morbid factor. However, if in addition to the morbid factor, other factors

that may produce a disease exist, then the resulting disease cannot be linked to the morbid factor alone. The other factors become co-morbid, and they may account for nearly all or some of the resulting disease state. Hence, co-morbid state may be defined as any factor that exists along with a main factor producing the disease state together. For example, the renal transplant patient may also be suffering from peptic ulcer or diabetes. These other diseases are potentially morbid and they are co-morbid with the chronic renal failure.

7.3 Methodologic Issues in Morbidity Studies

7.3 (i) Background

Morbidity is a complex phenomenon. It is difficult to measure. This difficulty is compounded by difficulties in interpreting morbidity data since the expression of morbidity is modified by social role and cultural values of the individual.[48] Reliability means the consistency between measurements in a series.[89] Illness is a disturbance of the relationships and feedbacks of three systems within each individual: the psychological system - the individual self - the perceptual system - which regulates a person's physical self and directs his/her behaviour, and the social system - which is the individual's relationship with the society.[48,49] These three systems reflect different manifestations of

morbidity: medical morbidity, perceived morbidity and social disability.

This chapter is restricted to the first type - medical morbidity among renal transplant and dialysis patients attributable to CMV infection. Morbidity is measured by collecting information about disturbance in the relevant system (in this case medical information). Although a particular study may be confined to one system, it is clear that the systems inter-relate and morbidity data must be interpreted with that understanding.

7.3 (ii) Reliability of Measurements in Morbidity Studies

To measure morbidity, indicators of morbidity must be identified. The reliability of such an indicator in measuring morbidity would vary according to the concept that is being measured, and the specific indicators chosen for such a measurement. Reporting of morbid symptoms is not always reliable even when the same individual repeats his/her report particularly if the condition is chronic.[48] In this study, morbid symptoms that would need reporting are not chronic. It is hoped that with a structured questionnaire the reported symptoms would be reliable. Provision would be made within the design of the study to check on the reliability of the reported symptoms.

7.3 (iii) Special Problems of Morbidity Statistics

Morbidity statistics is not as straightforward as mortality statistics in which death is a unique event. Illness (morbidity) may recur in the same person, and the cause may or may not be the same at different times. Death occurs at a point in time where as illness has a duration. Severity of illness may vary from time to time while the illness lasts. These differences must be taken into account where comparing morbidity statistics with that of mortality. These differences also make it more difficult to study morbidity.

Individuals may become ill more than one time during the study period. The question often arises whether the number of illnesses should be counted or the number of persons who are ill. In this study both the number of persons who are ill and the number of illnesses would be examined. It is essential to look at the number of persons who are ill because in comparing illness between the seronegative and seropositive individuals, it will be important to know how the illness is distributed among the members of each sample studied. If a few patients from a particular study sample have repeated illnesses, the sum of the illnesses for the sample from which those few individuals came may be so high as to regard that sample group as

showing a higher morbid state. Such a conclusion may, in fact, be erroneous.

7.3 (iv) Sources of Morbidity Statistics

(a) Survey of illness in which the study sample maintains a diary or are interviewed regarding their illness experience.

(b) General practitioner statistics: This source of information may not be very reliable, since patients go to their doctors for minor reasons very often.

(c) Hospital in-patient statistics are usually not very good for measurement of rates. Also, it is usually difficult to define the population from which the sample comes.

(d) Sickness absence record which indicates the number and dates of absence from work reflects the illness burden which indirectly is morbidity experienced by that population. This is true only if they usually go to work or school regularly.

(e) Notification of disease is another source of information. This is usually a very poor method of measuring morbidity since reportable diseases are not reported very often. The estimate based on notification of disease would therefore be far lower than the true situation. This method applies particularly to infectious diseases.

(f) Registration of all cases such as cancer or TB can be a useful source of data. In situations where interest is primarily on morbidity attributable to a specific agent, the notification, registration and survey type of case finding are useful sources of morbidity data. In this study, CMV is neither notifiable nor is a registry maintained for this infection. Therefore, the survey method becomes the method of choice in studying the morbidity attributable to CMV infection.

7.4.1 Diagnosis of Morbidity: Criteria

The diagnosis of morbidity may be set on a functional basis. For example, absent from work for more than one day a week whatever the cause of illness, could be regarded as significant morbidity for the particular sample of workers. On the other hand, morbidity may be related to medical standards such as abnormal temperature, heart rate, respiratory rate, jaundice, arthralgial and abnormal laboratory findings such as pneumonic process on chest x-ray, abnormal liver function tests and deteriorating renal function. Both approaches would be adopted in this study although the study leans to the medical standard more heavily than the functional approach. Neither the functional nor the medical standard approach has causal connotation. The primary interest of this chapter is assessment of degree of morbidity experienced by these renal transplant and

dialysis patients that can be attributed to cytomegalovirus infection. It is therefore necessary to link the observed morbidity with cytomegalovirus infection if any.

In this study, a patient would be said to be ill if he/she has one or more of the following indicators of morbidity:

(a) fever as defined in section 7.2 (iii),

(b) arthralgia as defined in section 7.2 (viii),

(c) respiratory signs and symptoms characterized by a rate $\geq$ 30/min., breathlessness, rales in the chest, intercostal and/or subcostal indrawing, positive radiologic findings in the chest as defined in section 7.2 (iv),

(d) jaundice as defined by yellowness of the sclera when observed by a physician,

(e) clinical and laboratory evidence of allograft rejection as defined in section 4.1. Also, oliguria ($<$ 200 ml of urine/24 hours) in renal transplant patients who have been passing over 1000 ml of urine previously; tenderness over the allograft and proteinuria of $\geq$ 500 mg/24 hours.

The association of any or all of the above features with cytomegalovirus is strengthened when it is supported by clinical and laboratory findings. The clinical findings of interest would be whether or not a focus of infection could be detected to explain the fever; whether what was reported as arthralgia is, in fact septic arthritis. When

such clinical findings are supported by laboratory findings, the strength of the association becomes even stronger. Therefore, laboratory findings such leukpoenia, viruria, seroconversion among seronegative patients and four-fold rise in antibody titer among previously seropositive patients become very important. Evidence of allograft rejection plus CMV isolation or rise in titer of antibodies as well as positive characteristic chest x-ray findings in the case of those who present with respiratory signs and symptoms also strengthen the establishment of causal association between the morbidity and cytomegalovirus infection.

7.4.2 Other Etiologic Factors

The observed morbid factors such as fever and arthralgia might be due to other infectious agents be it bacterial, viral or parasitic. It is therefore important to search for other organisms that may explain or contribute to explain the observed morbidity. For example, the patient could have coxsachie or echovirus infection presenting with fever, arthralgia and leukopenia. Early bacterial joint infection may be confused with virus infection. It is therefore essential to search for as many organisms as clinically reasonable, and to assess the contributions of such organisms to the observed morbidity. Chest x-ray, though relatively specific for CMV, does not exclude all other possible etiologic agents. Therefore, when a patient

presents with acute infectious process compatible with diagnosis of CMV, full septic work up (except lumbar puncture) and a search for other viral etiology would be undertaken.

7.5.1 Measurement of Morbidity

The renal transplant and dialysis patients live at home to a large extent (except at the time of renal transplantation) unless the patient is sick. Although the patients on centre (hospital based) hemodialysis have to come to the hospital two to three times a week (some, once a week) they do return home after each dialysis. Therefore, greater proportion of their time is spent outside the hospital. The transplant patients only come to the clinic once a month. They take their medications at home. Therefore, an instrument that can be used at home to identify morbid factors as defined in section 7.2.1 must be provided. For this study, instruments such as thermometer and a way of describing symptoms as set out in section 7.4.1 must be provided. Although the thermometer is precise in measuring presence and degree of fever, description of symptoms is not as precise. There will be a need to confirm the described symptoms and to add clinical evaluation in terms of physical examination (in addition to a review of the symptom history). Yet the physical examination is less precise than the laboratory assessment in etiologic diagnosis. The measurement of morbidity among these patients must of necessity take three

dimensions:

1. Field measurements,

2. Clinical measurements,

3. Laboratory measurements.

It is essential that the three are considered because they serve different functions in the diagnostic measurement processes. 1. Field measurements would elicit early symptoms. This is particularly so if the symptoms can appear, disappear and may or may not appear again; and yet the symptom is known to be associated with agents whose causal relationship to morbidity is our prime interest. An example is the sharp joint pain associated with cytomegalovirus infection among renal transplant patients. It is characterized by sudden stabbing pain involving the knees, hip, ankles, sacroiliac joints and wrist in that order of frequency, lasting 24 - 48 hours. This may not reappear and unless symptoms like this are monitored, important information about the morbidity attributable to CMV would be missed. Hence, there is a need for measurements at home (in the field).

2. Because of the limitations of the data generated through measurements at home by the patients (discussed below), there is a need for periodic clinical assessment at which time the data collected at home may be reviewed.

3. In assigning etiologic association to any viral infection, the laboratory measurements are crucial. Many viral infections could present with similar signs and symptoms.

7.5.2 Field Measurements

One of the major problems in morbidity study is for the subject to remember events that occurred in the distant past. It is known that while the subjects may remember major dramatic or disabling events fairly well over a month or two, it is not so for non-disabling events such as fever, small joint aches or cough without shortness of breath.[51] There is, therefore, a need for a monthly review of these symptoms in addition to a more frequent monitoring at home. A useful instrument at home is a health diary. This is a record of health events that the patient would record at the end of each day. This would be less subject to errors caused by lack of memory.[51] For this study, because some of the symptoms of CMV infection are mild, a daily diary would be particularly useful. There are limitations and shortcomings in the use of daily health diary. It is expensive and there may be problems of illegibility of the responder's entries which would make analysis and interpretation of the results difficult. The biggest advantage, however, is its ability to minimize non-sampling errors due to memory in situations where the

symptoms are not dramatic as is usual for CMV infection. It may reduce the problem of under reporting which normally results in low morbidity rates.

The household interview is one of the accepted methods of collecting morbidity data. The interview usually takes place in the homes of the subjects or at a predetermined location. The interview could take the form of a questionnaire which may be self-administered or administered by a trained interviewer. The respondent may be the subject or a substitute. Interview method of generating morbidity data has advantages and disadvantages. The primary advantage is the broadening of the observational base it permits while enabling one to conduct analysis with the individual rather than groups as the statistical units.[52] By applying modern knowledge of sampling design and techniques of eliciting respondents cooperation, it is possible to conduct interviews among a high proportion (often well over 90%) of the selected cases from a well selected sample representing a clearly specifiable population. This represents a clear cut advantage of the household interview over diary keeping. Another advantage that some investigators have noted is that household interview is cheaper than daily diary keeping.[51] However, the rate of under-reporting is quite high in interview based morbidity data unless the interview takes place within one month of the event or the event is disabling.

7.5.3. Sources of Errors in Diary and Interview Methods

(a) Accuracy of the Instrument:

In measuring accuracy of these instruments, account must be taken of the sampling errors. These are the errors that arise because samples rather than the total population are being examined. The sampling errors can be reduced by increasing the sample size. In our study, the cohort who are seronegative who are being watched for the development of morbidity due to cytomegalovirus constitute a sample from the population (unknown number) of CMV seronegative renal transplant patients.

(b) Account must be taken of the non-sampling errors. These include errors due to faulty definitions, to interviewers, the diary recorders, and where the subject is not the respondent, errors of memory and lack of knowledge of an event to which a question is addressed.

7.5.4 Interview Versus Diary Keeping in Morbidity Data Collection

One way of comparing interview techniques with diary keeping as a method of data collection in morbidity studies is to compare the ratio of rates generated through these methods. For example, episodes of illness, incidence of the condition, prevalence and days of illness can be

obtained by interview and by keeping daily diary for a particular condition. The ratio of the rates can be compared. An example was presented by Allen et al.[51] as shown in Table 7.2 (page 141).

In that study, diary elicited incidence rates were about twice the interview elicited rates. The ratio is close to unity for disabling illnesses. For non-disabling illness the difference is wide. This supports the data of Collins[53] that non-disabling illness will be forgotten more rapidly than the disabling illness. From these observations, one can suspect that diary keeping will minimize errors due to memory; and interview method is as good as diary keeping when dealing with dramatic or disabling conditions. The extra advantage of interview method specifically that of collecting data with wide observational base cannot be ignored.

For this study, both diary keeping and interview methods would be used. The diary keeping would be particularly useful to elicit early symptoms such as fever and arthralgia. This is important because most of the symptoms of CMV infections are usually mild. The purpose of the interview techniques in this case is to validate the diary records - checking with regards to completeness of the diary, and accuracy of the recordings.

TABLE 7.2

Ratio of Diary Rates to Interview Rates

Class of Illnesses	Episodes (A)	Incidence (B)	Prevalence (C)	Days of Illness (D)
All Illness	1.9	2.1	0.6	Not reported
Medically Attended	1.2	1.1	1.0	Not reported
Not Medically Attended	2.3	2.7	0.4	Not reported
Disabling Illness	1.8	1.8	0.4	1.0 1.0
Medically Attended	1.3	0.8	1.0	0.9 0.9
Not Medically Attended	2.0	2.1	0.7	1.3 1.3
Non-disabling Illness	2.1	2.4	0.5	Not reported
Medically Attended	1.0	1.3	1.0	Not reported
Not Medically Attended	2.5	2.8	0.3	Not reported

Diary Interview Ratios for Selected Rates of Illness, March-May 1952: San Jose Household Sample Survey: Allen et al.[51]

It is equally important that the questions for the interview be structured. This would give uniformity to both the interview and the response. The questionnaire carefully prepared in a dichotomous ("Yes" /"No") fashion can be used. This will also eliminate ambiguity in the response and it will minimize variations in the way the questions are framed.

The other consideration is "who would administer the questionnaire and how frequently?" There is evidence[51] to suggest that when interviews are administered within a month or at monthly intervals, the rate of eliciting the symptoms approximate that of health diary keeping. Therefore, these questionnaires would be administered on a monthly basis just before the clinical-laboratory evaluation. There are various ways of administering questionnaires in a household survey. A designated research assistant may visit the homes of individuals and administer the questionnaire. This is a relatively expensive method. However, the research assistant administering the questionnaire would ensure completeness of the questions. The completed information would be easier to compare. The third method is a self-administered questionnaire. This method is applicable to the present study. It is cheap and convenient. This questionnaire could be completed about a week before the patient attends the monthly clinic for clinical-laboratory

evaluation. It is important that the patient cooperate as far as completing the questionnaire is concerned. He/she must be prepared to set aside about fifteen minutes to answer the questions. It is preferred that the patient does not look at his/her diary before completing the questionnaire.

7.5.5 Self-Administered Questionnaire: Its Reliability

The use of self-administered medical questionnaire has many advantages. It has been used to conserve physicians' time. It is economical and it permits some standardization, uniformity of administration and minimal interviewer's bias (Collen et al.).[72] The experience of Collen et al.,[72] Abrahamson et al.[73] indicates that self-administered questionnaire is reliable. The ultimate hope is that the questionnaire is a valid instrument for measuring medical conditions.

Reliability is defined as the consistency (reproducibility) with which the instrument elicits the same response. If the same question is asked the second or third time, if the responses are the same, the questions (instrument) is reliable. Without a reliable instrument, the question of validity of the response does not exist. In other words, if each time a question is used to elicit a fact, different responses are obtained, it is difficult to believe any of the responses as the "correct one". Validity can be defined as the ability of a question to secure the desired information

such as to obtain the correct history of a specific condition (eg. a question directed towards eliciting the amount of weight lost in the past year is valid if it produced an accurate response).[72]

Since medical questions are primarily directed toward eliciting factual information, whatever instrument (questionnaire in this case) used must be examined for its reliability and validity. Because past information cannot be validated and future information is gathered so slowly, validity of medical questionnaire is difficult to evaluate. Reliability of the instrument is easier to determine. If the instrument is reliable, the investigator has some assurance towards validity of the instrument that is being used.

Test of Reliability of a Questionnaire

To test reliability (reproducibility) the questionnaire has to be administered twice, at least. The individual may be given the questions on a regular type and be allowed to respond to the questions. About an hour later, he may be given the same questions, but now they would have been printed on cards with different colours. The patient is again allowed to respond to the questions. Given that the fact to be elicited is constant and is related to the respondent, the response should be the same as before if the question is clear enough to elicit the fact. Where yes or

no answers are intended, a 2 x 2 type of table can be generated to summarize the response of many individuals using the same question twice. The changes that may be observed in the response would be "Yes" to "No", and "No" to "Yes", as displayed in Table 7.3 (page 146).

Each of the questions would then be evaluated for individual changes between the first and second response. The percentage change from "Yes" to "No" is computed from C/A + C, and the percentage change from "No" to "Yes" is also computed from B/B + D. The questions can be ranked into decreasing order of reliability. It is important to take into account the overall "Yes" response to a question in order to appreciate the significance of a change from "No" to "Yes". If a very small proportion initially answered "Yes" to a question and a relatively large percentage changed from "No" to "Yes" on that question, it may not be significant. On the other hand, if a large proportion answered "Yes" to the question to begin with, and then the same changed from "No" to "Yes" on that question, the question is relatively reliable.

Therefore, adjustment has to be made for the varying proportions that answered "Yes". A relative percentage could be computed (R) as illustrated by Collen et al.[72] This is achieved by dividing the absolute percentage change from "No" to "Yes" by percent, who answered "Yes" on the first

TABLE 7.3

RESPONSE PATTERNS

		Answer on First Test		
		YES	NO	TOTAL
Answer on Second Test	Yes	A (Yes → Yes	B (No → Yes)	A + B (Yes or No → Yes)
	No	C (Yes → No)	D (No → No)	C + D (Yes or No → No)
	Total	A + C (Yes → Yes or No)	B + D (No → Yes or no)	A + B + C + D (Yes or No → No or Yes

test:

$$R = \frac{B/B + D}{A + C/A + B + C + D} = \frac{B}{B + D}\left[\frac{A + B + C + D}{A + C}\right]$$

$$= \frac{\text{"no"} \longrightarrow \text{"yes"}/\text{"no" first time}}{\text{"yes" first time/total}}$$

$$= \left[\frac{\text{"no"} \longrightarrow \text{"yes" first time}}{\text{no}}\right]\left[\frac{\text{total}}{\text{"yes" first time}}\right]$$

To derive a score for each question with equal weight to both types of change, the maximum percentage changes for "yes" "no" is"

$$\left(\max \frac{C}{A + C}\right) \quad \text{and} \quad \left(\max \left[R\right]\right)$$

were computed respectively. Then, for each question, the percentage change of each type is divided by one max, i.e.:

$$\left[\frac{C}{A + C} \div \max \frac{C}{A + C}\right] \quad \text{and} \left[R \div \text{Max } (R)\right] \text{respectively.}$$

Hence, scores of 0 to 1 were obtained for each questions's "yes" to "no" and "no" to "yes", which added to 0 to 2. The questions are then ranked with increasing magnitude of score. The bigger the score the less the reliability of the questionnaire. From the studies of Collen et al.,[72] it was evident that the use of precise words, avoidance of ambiguous terms, avoidance of answering the questions when the respondent is fatigued or is in any

physical discomfort would enhance the reliability of the questionnaire. It is advisable to test the questionnaire for reliability at the inception of the study. In Collen's study, it was shown that for the 95% who answered "no" the first time to their best question, less than 0.5% changed their response on retesting. That question is certainly reliable.

7.6.1 Clinical Evaluation

The patient would be seen at the regular clinic on monthly basis. In the clinic, complete history for the month would be obtained. Particular attention would be given to symptoms referable to infectious process. A complete physical examination would be done. The clinician would be blind to both daily health diary and the monthly questionnaire results until he has recorded his findings. Details of the findings would be recorded. The clinician would then review both the daily diary recordings and the completed questionnaire. The physician will be blind to whether this patient is seronegative or seropositive.

7.6.2 Laboratory Evaluations

This would include general routine laboratory tests as well as serology and virologic tests for CMV. Details of the tests are described in section 7.9.5. The laboratory staff will be blind to the clinical condition of the patient.

7.6.3 Functional Approach to Morbidity Data Generation

Various functional variables could be used to measure morbidity. Because the study is concerned with medical morbidity the greater proportion of the instruments used for measuring morbidity are medical. However, functional capacity of the patient is also a good measure of morbidity the patient experiences. Functional instrument does not have causal connotation.

Bed days is one of the measures that some investigators have found reliable as an instrument.[97] Bed days has been defined in section 7.2.1. This measure would be used in the present study. Restricted activity days as defined in section 7.2.1 would also be used to measure morbidity.

7.7 Overall Design of the Experiment to Study Morbidity Among Renal Transplant and Dialysis Patients

(i) Both the seronegative and the seropositive pre-transplant patients who are admitted for the study would be included in the morbidity study.

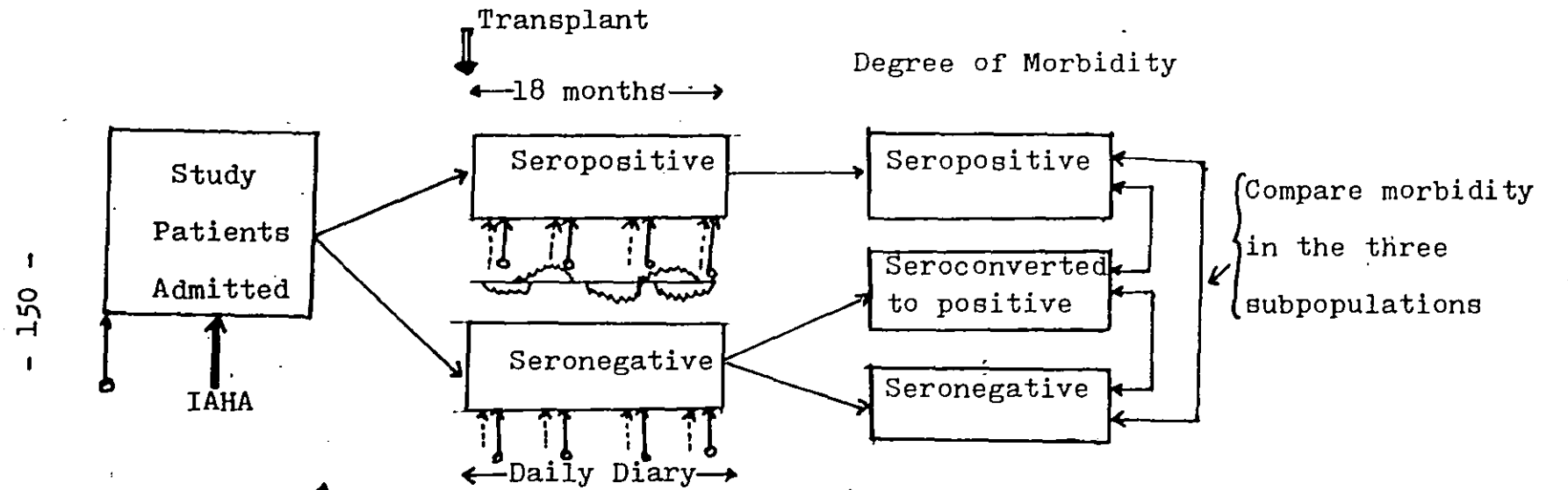
(ii) The overall plan for the morbidity study would be presented as shown diagramatically in Figure 7.1 (page 150).

7.8 Study Population

For morbidity study among renal transplant and dialysis patients, the total sample that was admitted into

FIGURE 7.1

The Overall Experimental Design to Study Morbidity and Mortality Among Renal Transplant and Dialysis Patients



Key: = monthly questionnaire; = monthly clinical-laboratory evaluation;

= acute episodes evaluated comprehensively in terms of IAHA, urine and throat swab for cultures, liver function tests, BUN, creatinine, chest x-ray, blood culture, etc.

the study as specified in section 4.4 would participate. When originally the IAHA test was used to test the serum of these patients, they were separated into seropositive and seronegative groups. The pretransplant seropositive patients would be included although the time of their seroconversion would be unknown. They would be regarded as having silent CMV. They form an interesting group to be compared with those who become seroconverted post-transplant in terms of morbidity experience. The seronegative individuals would also be followed for morbidity. It is expected that this group may experience morbidity due to other causes but CMV. If there is excess of morbidity among the other two groups above the seronegative group, that excess may turn out to be CMV related. Only the renal transplant and hemodialysis patients would be followed. The size of each group would be as outlined in section 4.2.

7.9 Details of the Morbidity/Mortality Study Among Renal Transplant and Dialysis Patients

7.9.1 Introduction of the Patients to the Study

When the patient is found suitable for the study on the basis of the admission criteria as outlined in section 4.4 (i), the whole purpose, plan and progress of the study is explained. The patient is then invited to participate and informed consent is sought by the responsible physician. Patient is then introduced to the daily health diary, the

questionnaire forms and he is given a thermometer, the use of which is explained and demonstrated to him. The patient is instructed on the importance of proper shaking of the thermometer to zero before use each time. The fact that a research coordinator is available freely at any time is made known to the patient. The research coordinator serves as a resource person and he/she is the one to whom important symptoms must be reported through the patient's physician. The essence of the clinical and laboratory evaluation is explained to the patient.

7.9.2 Details of the Study

This portion of the protocol pertains to the individual patient who is participating in the study but living at home. The daily health diary is designed in such a way that there is enough space for comments and short descriptions of the symptoms. The diary becomes the personal property of the patient. It is started on the first day the patient enters the study and lasts through 18 months, during which time the patient would be in the study. By the use of the diary, the patient is instructed to record every fever (greater than 38°C). Whenever the patient feels unwell and/or feverish, he/she is expected to check the temperature. Symptoms such as joint pain, cough, difficulty in breathing should be recorded. Short descriptive notes are encouraged. In a day, the patient may have fever, joint pain and cough.

All these symptoms must be reported to the research coordinator through the patient's regular physician. It is important also that such symptoms as anorexia, nausea or a general unwell feeling be reported. It is suggested that the diary be filled at the end of each day. In order to improve compliance in filling the diary, the patients may be told that the diary is to be completed just before the patient's regular medication. As long as the patient accepts the routine of diary before drug, the compliance is likely to remain good. End of the day is chosen for filling the diary to eliminate having to fill in two symptoms occurring at different times of the day, twice during the same day.

Major symptoms (characterized by high (T = 39°C) termperature, and/or arthralgia with sudden onset, stabbing in character or rapid breathing, shortness of breath, chest pain and fever) demands that the regular physician be contacted immediately. The physician will notify the research coordinator; the coordinator would arrange for the patient's clinical and laboratory evalution. Also, the above symptoms, singly or in combination, are major as far as CMV infection is concerned. The patient should report all other symptoms (illnesses) to the coordinator whether the illness appears relevant to CMV or not. During the period of the study, the study team assumes total care of these

patients, except technically specified care such as adjustment of drugs for the anti-rejection purposes or changes in the hemodialysis schedule.

7.9.3 A week prior to the monthly clinical evaluation of the patient, an envelope that has been mailed to the patient sealed (containing questionnaire about the patient's health in the previous four weeks) is opened. The questions are answered completely. Patient is encouraged to give himself enough time to answer all of the questions at one sitting. Patients are encouraged not to look at their daily diary. The completed questionnaire is then sealed back in the envelope provided. This envelope is taken along to the clinic.

7.9.4 Visit to the Clinic

Once a month the patient visits the clinic for evaluation. The purpose of the visit includes:

(a) A check on the compliance of the study patients regarding keeping the diary properly and completely filled in; the proper and completeness of answering the questionnaire;

(b) A clinical evaluation which includes interim history and physical examination without reference either to the diary or the questionnaire is performed. The physician does not know whether the patient has been seropositive, is seroconverted or remains seronegative;

(c) Laboratory evaluation: The monthly visit affords the opportunity to take blood for serology and essential biochemical tests that need to be followed in any renal patient such as BUN, creatinine.

The first evaluation at the beginning of the study is unique. Complete, comprehensive history and physical examination is done. This includes:

1. CBC and differential, platelet counts,
2. BUN, creatinine, electrolytes, calcium, phosphorus, uric acid,
3. SGOT, SGGT, alk phosphotase, bilirubin, LDH,
4. Chest x-ray,
5. Throat swab, urine for CMV culture,
6. Blood for IAHA tests for CMV antibodies.

7.9.5 Acute Illness of Infectious Nature of Rejection of Allograft

When any of the study patients have major symptoms as described above, the patient must be seen, evaluated thoroughly and etiologic agent responsible for the symptoms must be sought. In addition to regular thorough history and physical examination, the physician must pay particular attention to the respiratory system, the joints (looking for evidence of arthralgia or arthritis) and carefully palpate the liver. Then, laboratory investigations must be used to aid the search for etiology. CBC and differential,

platelet count, sedimentation rate must be done. Also BUN, creatinine, uric acid, electrolytes, calcium, phosphorus, would be done. Liver function tests such as SGOT, SGGT, alkaline phosphotase, LDH and bilirubin would be done. If there is evidence of allograft rejection, then IVP, renal scan and/or renal biopsy would be done. Urinalysis and urine culture must be done. In search for possibility of CMV as etiologic association with the illness, the following tests would be performed:

(a) Urine and throat swab for culture for CMV,

(b) Blood culture for CMV,

(c) If renal biopsy done, then a piece of the tissue for viral culture,

(d) Blood culture for bacteria,

(e) IAHA tests for CMV antibodies,

(f) Chest x-ray,

(g) lymphocytes from patient for blastogenesis

(N.D. - the serological tests would include acute and convalescent tests run in parallel).

7.10.1 Criteria Set for Clinical Evaluation

In order to give uniformity to the data and to make comparison easy, certain guidelines for clinical evaluation are important. The clinician has to pay particular attention to specific systems and quantify, if possible, any abnormality that may be detected. For this study:

Lymphadenopathy would be graded 0, 1+, 2+, 3+. These are arbitrary gradings where 0 indicates no lymphadenopathy, 1+ = few (within normal range), 2+ = moderate and 3 + = gross lymphadenopathy. Respiratory rate would be rated as 18 - 30 = 1+ + normal, greater than 30 = 2+ = abnormally high.

Respiratory pattern: Indrawing of intercostals, subcostal and suprasternal would be graded 0 = none, 1+ = mild, 2+ = moderate and 3+ = severe.

Hepatic enlargement which may point to hepatitis would be similarly grade 0 = no enlargement, 1+ = slightly enlarged and 2+ = grossly enlarged. Joints would be assessed for tenderness: 0 = no tenderness, 1+ = slight tenderness, 2+ = tenderness (moderate, not warm), 3+ = quite tender and warm = severe. Similarly, joints would be assessed for range of movement. 0 = full range of movement, 1+ = slight limitation in movement of the joint, 2+ = moderately limited and 3+ = severely limited in the range of movement.

Fever would be scored: 37 - 38°C = 0 = normal; 38 - 39°C = 1 = low grade pyrexia; 39 - 40°C = 2 = high fever. The clinical who would be scoring these patient must be consistent in what he calls 0, 1+, 2+, 3+ as the case may be. Reproducibility of his scoring system is very cruicial to the validity of the clinical data. In order to minimize the sources of variation, only one

physician would evaluate the patients clinically. Specifically for joint evaluation, because the findings are likely to be subtle in cases of CMV infection, it is important for the clinician to try and evaluate and score joints of patients with mild arthritis prior to the study. That exercise would help him to realize how reproducible his scoring is and he would improve on the variations of his scores.

Criteria for Laboratory Evaluation

For this study, WBC less than 4500 would be regarded as leukopenia. The criteria defined in section 7.2.1 would be adhered to.

7.10.2 Questionnaire

It is important to give uniformity to the questions answered by the patients so that meaningful comparison can be made between and within patients. To that end, a questionnaire is designed. It is equally important that there is no ambiguity in the answers the patients can give. Therefore, "Yes" or "No" response is required as answers. It is even more important that the questions are not ambiguous, that the questions are clearly stated and that the questions would be understood by all. This latter problem is not easy. It is hoped that the questions below meet those specifications.

For cytomegalovirus, the main systems that are involved that manifest clinical symptoms include:

(a) respiratory;

(b) musculoskeletal system (mainly joints) and systemic response in the way of fever and generalized malaise. If there is hepatitis, nausea, anorexia and vomiting may be present. These questionnaires are, therefore, addressed to these systems mainly. The questionnaire on the respiratory system took very much after the one designed by Fairbairn et al.[74] (Questionnaire - pg. 160.)

7.10.3 Data Management

Data management is an important aspect of the study. Data may be stored on flow sheets or in the computer. The method of choice depends on:

(a) the size of the data;

(b) the availability of a computer with appropriate programs;

(c) the availability of skilled personnel to manage the data in the computer. The use of computer for data management is efficient especially if the data is large. The use of computer to store the data gives great flexibility in that data can be retrieved, questions can be asked and partial analysis can be done quickly while the study is still in progress. On the other hand, the operation of the data in

QUESTIONNAIRE

Name: _____

Address: _____

Date of birth: _____

Record number: _____

Primary Diagnosis: _____

Phone number: _____

		Yes	or	No
1.	Do you usually have a cough?	____		____
2.	Do you cough at all when you get up in the morning?	____		____
3.	Do you go on coughing during the day? . . .	____		____
4.	Do you produce phlegm in the morning? . . .	____		____
5.	Do you bring it up during the day?	____		____
6.	What colour, if you bring up phlegm?			
	always clear white	____		____
	occasionally yellow or green .	____		____
	usually yellow or green	____		____
7.	Grade 1: Are you ever breathless, except on exertion?	____		____
8.	(If yes) Grade 2: Are you short of breath when hurrying on level or walking a slight hill?	____		____
9.	(If yes) Grade 3: Do you have to walk slower than most people on the level? Stop after a mile? (moderate)	____		____

		Yes	or	No
10.	(If yes to either) Grade 4: Do you have to stop for breath after walking 100 yards?	____		____
11.	(If yes) Grade 5: Are you too breathless to leave the house?	____		____
12.	During this month, have you had any sudden change in your breathing pattern?	____		____
13.	Was the change associated with cough? . .	____		____
14.	Was the change associated with fever and cough?	____		____
15.	How long did it last? 1 day	____		____
	2 days	____		____
	3 - 5 days	____		____
	1 - 2 weeks	____		____
16.	Was there any association with sputum production?	____		____
17.	Was there any pain accompanying the change in respiratory pattern?	____		____
18.	Do you have arthritis?	____		____
19.	For how long have you had arthritis?			
	Recently?	____		____
	Long ago?	____		____
20.	Do you usually have joint pains?	____		____
21.	Do you usually have stiffness of joints in the morning?	____		____
22.	Do you take medicine for your joint problem?	____		____
23.	If you have never had joint problem prior to this study, this month have you had			
	joint pain?	____		____
	swollen joints?	____		____
	joint stiffness in a.m.?	____		____

Yes or No

24. Did you have any fever this month? . . . ____ ____

25. Were you sick at all this month to a point where you could not do your normal duties? ____ ____

26. Did you stay in bed for a whole day? . . ____ ____

half a day? . ____ ____

less than half a day? . ____ ____

not at all? . ____ ____

Times					
	1	2	3	4	4+

27. Were you so ill this month that you reduced your activity to

half your usual . ____ ____

one quarter . . . ____ ____

whole day . . . . ____ ____

less than half a day . . ____ ____

Times					
	1	2	3	4	4+

the computer demands availability of skilled personnel. If the computer breaks down, there must be someone, who can deal with the problem, available. There is also a large amount of time and expense required to design and write the programme necessary to effectively use the computer. Therefore, the cost of using the computer may be high for the advantage one derives from it.

The use of carefully prepared flow sheets for each patient may be more desirable if the data is not too large. The use of flow sheets is simpler and cheaper. Anybody can enter data into it, look at the data from the sheets and retrieve the data by retrieving the flow sheets without difficulty.

For this study, carefully prepared flow sheets for each patient is preferred. If there is any need for data analysis before the conclusion of the whole study, the data from the flow sheet can be fed into the SPSS computer soft ware packages with specific statistical analysis done, depending on the questions that arise. At the conclusion o f the study, the data will be reviewed by the statistician and appropriate tests to answer the original questions would be performed.

Flow sheets would be in two forms: (i) sheets that contain constant (relatively) information, and (ii) forms that contain changing information:

Form I: Name
Age
Sex
Original diagnosis
Dialysis (d) or Transplant (T)

Form II: IAHA titer
Hepatitis B (positive/negative)
Biochemical tests
Hematologic tests
Virologic tests
Clinical information
Treatment modality
Immunologic information

The forms to be used for x-ray reports, serologic reports (including the raw data), biochemical, hematologic and microbiologic tests will be designed to accommodate the actual observed results unprocessed (such as averaging). These raw data will be processed and transferred to the patient's all-purpose flow sheet. The raw data will be kept as well. Information from daily diary and the monthly interviews would be kept along with the clinical assessment information. Both of these would form the clinical information on the all-purpose flow sheet for each patient. A secretary/clerical staff will be in charge of updating the all-purpose flow sheet. Once a month the secretary/clerical staff will review the data of all patients as on the all-purpose flow sheets with the research coordinator to ensure that all flow sheets are up to date. Missing data must be entered immediately. At the conclusion of the study, and before the data analysis is begun, the all-purpose flow sheet

of every patient would be duplicated. The duplicate would be kept by the research coordinator. The original copy may then go to the statistician who would also have access to the raw data (kept by research coordinator). A draft of all-purpose flow sheet is shown on page 166.

ALL-PURPOSE FLOW SHEET

Name:

Sex:

Age:

Original diagnosis:

Dialysis (D) or Transplant (T):

Key: SGOT, SGGT - liver function tests
X-ray grades - 0 = normal, 1,2,3,4 = abnormal
Cough - 0 = none, 1,2,3 = mild, moderate and severe
Joint pain - 0 = none, 1,2,3, = mild, moderate or severe.

Date					
IAHA (titers)					
Hepatitis B (positive/negative)					
Na, K, Cl^-					
BUN					
Creatinine					
SGOT					
SGGT					
Bilirubin					
WBC (white cell count) (total)					
Neutrophils (%)					
Lymphocytes (%)					
Bands (%)					
Monocytes (%)					
Hemoglobin (gm/dl)					
Respiratory rate 30/min. at rest					
Cough (more than occasional)					
Rales in Chest					
Chest x-ray					

Fever (T $\geq$ 38°C) - report highest					
Painful joint - ankle					
knee					
hip					
elbow					
wrist					
Urine for CMV (positive/negative)					
Throat swab for CMV (positive/negative)					
Other infectious agents					
Rejection episodes					
Immunoglobulins					
Lymphocyte stimulation index					
Immunosuppressant treatment (Yes or No)					
-name of drug					
-dose of drug					
Bed - days/2 weeks					
Restricted activity - days/2 weeks					
Bed days +/- restricted activity = physician contact for ill health					

7.11 Dummy Tables for Data Analysis

For Groups I - seropositive at the inception of the study.

II - seronegative to start with, then seroconverted during the study period.

III - remain seronegative.

These groups would be compared with respect to degree of morbidity, types of morbidity, chronologic onset of morbidity, if there is any relationship with primary diagnosis, sex and effects of co-morbid factors. (Tables 7.4 and 7.5)

TABLE 7.4

Group	N	Rejection Episode	Hepatitis	Pneumonia	Fever	Arthralgia
I						
II						
III						

TABLE 7.5

Group	Rejection	Hepatitis	Pneumonia	Arthralgia	Fever	Bed Days	Restricted Activity	Total
I								
II								
III								

CHAPTER EIGHT

8.1 Data Analysis

For this study, there are three components viz: incidence, morbidity, cellular immunity. The investigators may want to raise questions such as those below. The questions pertaining to incidence study are:

(a) What is the incidence of CMV among renal transplant patients?

(b) What is the incidence of CMV among dialysis patients?

(c) Is there any significant difference between the incidence of CMV among the transplant and dialysis patients?

(d) Is there any relationship between primary diagnosis and incidence of CMV among these patients?

(e) Are there sex differences in the incidence of CMV?

(f) Is there any significant association between source of kidney, blood transfusion and incidence of CMV?

For this study IAHA titer of $< 1:8$ is regarded as negative and $\geq 1:8$ is positive. The positive cases can be counted and expressed as a fraction of the total patients in the study. This is the prevalence of the CMV in the study population. When the cohort of seronegative patients

are followed for the 18 months; the resulting seroconversion creates new cases that are positive. The fraction of the new positive to the total original negative is incidence.

It is conceivable that the investigators may want to examine the high titer positives aribtrarily set at titer $\geq$ 1:128 and low titer $\leq$ 1:64 and see if the primary diagnosis, the duration of dialysis, the amount of blood transfused are significantly associated with low or higher titer. All the tests so far can make use of χ^2 since they are frequency counts. Example: Question: What is the incidence of CMV among renal transplant patients?

$$\text{Incidence rate} = \frac{\text{No. of new cases per year/1000}}{\text{Total study sample}}$$

Let n = total no. of seronegative at beginning

x = seroconverted to positive so that (n - x) remain seronegative.

$$\therefore \text{Incidence} = \frac{x}{(x) + (n - x)} = \frac{x}{n}$$

In this case, duration of study = 18 months. To reduce the incidence to per year multiply by 2/3 (12/18)

$$\therefore \text{Incidence} = \frac{2x}{3n} \text{ per year.}$$

This applies to trasnplant or dialysis patients. For the questions of significant difference between incidence of CMV among renal transplant and that among

dialysis patients the data problem is proportion to serconversion. χ^2 statistics will be applied.

Association between primary diagnosis and incidence of CMV can also be assessed by χ^2 test. The general layout of the data can be represented as shown below.

TABLE 8.1

	Transplant	Dialysis	Total
Seropositive	a	b	e
Seronegative	c	d	f
TOTAL	h	g	N

$$\chi^2 = \frac{(|ad - bc| - \frac{1}{2}N)^2 N}{efgh}$$

8.2.1 Morbidity Data Analysis

For overall assessment of morbidity among the always seropositive, the seroconverted, and always seronegative patients, the morbidity will be scaled to "scores" which vary according to severity of morbidity. The morbid factors under assessment include:

(a) Chest infection

(b) Pyrexia of undetermined cause

(c) Hepatitis

(d) Arthralgia

(e) Allograft rejection among transplanted patients

(f) Social dysfunction: (i) Bed days

(ii) Activity restricted days.

These morbid factors will be categorized into:

(i) none,

(ii) mild,

(iii) moderate

(iv) severe depending on the severity of the signs, symptoms and laboratory results where applicable.

Scores ↓ = decrease; ↑ = increase

(a) Chest infection: (i) = non infection = 0

(ii) temperature $\leq 38.5^{\circ}C$
respiratory rate ≤ 30/min.
$\pm$ rales in chest
+ radiologic suspicion
or minimal infiltrate
} mild = 1

(iii) temperatures = $38.5\text{-}39^{\circ}C$
respiratory rate = 31-35/min.
+ rales in chest
+ definite infiltrate on chest x-ray
} moderate = 2

(iv) temperature > 39°C
respiratory rate > 35/min.
rales in chest
definite radiologic
infiltrate on chest x-ray
} severe = 3

(b) Pyrexia of undetermined cause

All other causes but CMV negative

(i) temperature ≤38.5°C for >24 hours = mild = 1

(ii) temperature 38.6-39°C for 24 hours = moderate = 2

(iii) temperature > 39°C for 24 hours = severe = 3

(c) Hepatitis: (i) Generalized malaise for 3-4 days
± hepatomegaly
+ increased serum bilirubin ≤1.9 mg%
+ ↑ SGOT by less than 50%
+ ↑ SGGT by less than 50%
} Mild = 1

(ii) Generalized malaise for 3-4 days
± hepatomegaly
± jaundice
+ ↑ bilirubin 2-2.5 mg%
+ ↑ SGOT by <100% >50%
+ ↑ SGGT by <100% >50%
} Moderate = 2

(iii) Generalized malaise as above
± hepatomegaly
± jaundice
+ ↑ bilirubin
+ ↑ SGOT by ≥ 100%
+ ↑ SGGT by ≥ 100%
+ ↑ alkaine phosphatase

Severe = 3

(d) Arthralgia (i) none = 0

(ii) sudden pain in joint less than 12-24 hours
noticeable pain but not activity limiting
no swelling or redness

Mild = 1

(iii) Sudden pain in any joint lasting 24-48 hours
noticeable and activity limiting but not incapacitating
required mild analgesic

Moderate = 2

(iv) sudden pain in any joint lasting ≥ 48 hours
noticeable and markedly incapacitating requiring analgesics and medical attention (ASA)
± swelling at the site of pain

Severe = 3

(e) Allograft rejection: (i) no rejection = 0

(ii) ↑ creatinine ≤ .2 mg%/ 24 hours
↓ urine output by ≤ 30% previous 24 hours
generally unwell
clinical ± biopsy
± scan
diagnosed rejection
] Mild = 1

(iii) ↑ creatinine by 0.2-.5 mg%/ 24 hours
↓ urine output by 30-50%/ 24 hours
generalized malaise
± tender allograft
clinical rejection ± biopsy
± scan
] Moderate = 2

(iv) ↑ creatinine by ≥ .50 mg%/ 24 hours
↓ urine output by > 50% of previous 24 hours
± tender allograft area
clinical rejection ± biopsy
± scan
] Severe = 3

(f) Bed days: (i) none = 0

(ii) $\leq$ 3 days in any one month = mild = 1

(iii) 3 - 5 days in any one month = moderate = 2

(iv) $>$ 5 days in any one month = severe = 3

Restricted Activity Days:

(i) none = 0

(ii) $\leq$ 5 days in a month = mild = 1

(iii) 5 - 10 days in any one month = moderate = 2

(iv) $>$ 10 days in any one month = severe = 3

(g) Leukopenia: Mild = 4500 - 500/cu. mm WBC = 1

Moderate = 3000 - 4500/cu. mm WBC = 2

Severe = less than 3000/cu. mm WBC = 3

Each of the mild, moderate and severe should be accompanied by greater than 2% atypical lymphocytes.

The overall morbidity data can be presented as in Table 8.2 on page 178.

Since the total number that fall within the categories of mild, moderate and severe are counts (frequency), χ^2 can be applied to the whole data. The χ^2 value can then be partitioned in order to be more specific if there is any significant difference between the groups. The table for χ^2 will then look like Table 8.3.

The next task in the analysis is to relate the scores to CMV isolation and serologic tests. For this task

TABLE 8.2

Group I - Always CMV seropositive

Group II - Seroconverted to positive

Group III - Always CMV seronegative

	I			II			III			
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe	Total
Chest infection										
Pyrexia										
Hepatitis										
Arthralgia										
Allograft rejection										
Leukopenia										
Bed days										
Restricted activity days										
TOTAL										

Overall morbidity data will be presented in a table as shown above.

TABLE 8.3

	Mild	Moderate	Severe	Total
Initially and always seropositive	a	b	c	a + b + c
Seroconverted to positive	d	e	f	d + e + f
Always seronegative	g	h	i	g + h + i
TOTAL	a + d + g	b + e + h	c + f + i	N =

This is a 3 x 3 table with (3-1)(3-1) = 4 degrees of freedom

the always seronegative do not come into the analysis. They are presumed free from CMV infection. For the seroconverted (Group II) the time of seroconversion, the serologic titer and the morbid factors need to be related to establish association between the morbidity and CMV infection and/or allograft rejection. The diagnosis of CMV by serology $\pm$ viral isolation can be set against the morbid factors for Group II as shown in table 8.4 on page 181. This is a 2 x 8 with (2-1)(8-1) = 7df. χ^2 can be used to test if there is any association between these morbid factors and the diagnosis of CMV infection. The χ^2 value if significant can be partitioned. The same analysis can be done on the Group I data and the χ^2 values compared with respect to the degree of association by using phi = Φ =

$= \sqrt{\frac{X^2}{n}}$ = coefficient of association.

It is important also to know whether there is an association between seroconversion and the primary diagnosis because if there are particular diagnostic groups that have high rate of seroconversion and if it is established that seroconversion predisposes to morbidity, patients with such primary diagnoses may form a high risk group. In order to examine the association between the primary diagnosis, CMV infection and morbidity, a three level of variables must be constructed. Although these are counts and scores the simple χ^2 test cannot be applied. Suppose there are five

TABLE 8.4

Morbid Factors	Serology +ve Alone	Serology + Viral Isolation	Total
Respiratory infection			
Pyrexia			
Arthralgia			
Hepatitis			
Allograft rejection			
Leukopenia			
Bed Days			
Restricted Activity Days			
TOTAL			

Analyse the data by χ^2 with (8-1)92-1) = 7df.

diagnostic categories (five primary diagnoses), a table can be constructed as shown on page 183 (Table 8.5). This is a 2 x 3 x 5 table. The data can be analysed using a log-linear model. This model will enable the analyst to isolate not only the main factors of association but also the interaction terms can be isolated (see Appendix II).

By analysing the data in terms of groups only, useful information may be missed. It is important to count the number of people in each group who experienced morbidity. It is equally important to count the number of morbid episodes experienced by each group. If few people are sick frequently in a particular group, then there will be a wide difference between the results of analysis by counting those who were sick as compared to counting the sickness experience. Just a few frequently sick individuals in a particular group may therefore lead the analyst to a wrong conclusion unless note is taken of such a possibility.

8.2.2 Co-morbidity

The co-morbid factors will be scored at the functional level. If a condition of ill-health co-exists but does not reduce the functional capacity of the individual the score is 1 and that category is regarded as mild. On the other hand, if the condition is bad enough to reduce top level activity to 1/3 to 1/2 the score is 2 and the factor

TABLE 8.5

Diagnoses ↓ Morbidity →	CMV Positive			CMV Negative			
	None	Low	High	None	Low	High	Total
Glomerulonephritis							
Hemolytic Uraemic Syndrome							
Polycystic Kidney							
Phenacetic Nephropathy							
Pyelonephritis							
TOTAL							

This is a 2 x 3 x 5 table. The data can be analyzed by log-linear statistics.

is moderately severe. If the limitation is ⩾ 50% then it is severe and the score is 3. These scores will relate to co-morbid conditions such as peptic ulceration, asthma, ulcerative colitis, epilepsy, diabetes, rheumatoid arthritis, etc. co-existing with the advanced renal disease for which the study patients are in transplant or dialysis programmes.

The effect of co-morbid factors on the overall morbidity for each group will be presented as shown in Table 8.6 on page 185. The data can then be analysed by log-linear statistics.

8.3 Analysis of Immunity Data

The lymphocyte stimulation test results will be in the form of mean counts. These tests will be done at the inception of the study and at the time the patients are ill. Immunoglobulins would be done about the same time. Therefore, there will be data on the immunoglobulin levels as well as the lymphocyte stimulation tests at:

(a) initial

(b) seroconversion (0, 2, 4 weeks)

(c) 3 months, 6 months and at the conclusion of the study.

Therefore, the possibility of six or seven tests in an individual exists. The results of the mean counts as well as the immunoglobulins for always seropositive group would be compared with those of seroconverted and seronegative

TABLE 8.6

	I			II			III		
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe
Diabetes									
Arthritis									
Peptic ulcer									
Epilepsy									

Co-morbid factors versus morbidity in groups.

3 x 3 x 4 table.

N.B. This table could be 3 x 3 x C where C is the number of co-morbid factors of interest. A log-linear model is appropriate technique for the analysis of the data.

groups. As groups, the results of lymphocyte stimulation tests can be represented as shown in Table 8.7

TABLE 8.7

Time	Group I	Group II	Group III
Initial			
Seroconversion → 0 time			
Seroconversion → 2 weeks			
Seroconversion → 4 weeks			
3 months			
6 months			
End point			

This will be tested by analysis of variance. If there is any tendency to linear trend in Group II, this would be tested by regression analysis.

Regarding Table 8.8 on page 187, this is a 3 x 7 x 5 table for analysis of immunologic data. The data in a 3 x 7 x 5 data will be analysed by a log-linear model as programmed in ECTA (computer). Also the data of the lymphocyte stimulation index will be regressed on the immunoglobulins to explore the possibility of linear trends between immunoglobulins (humoral immunity) and lymphocyte stimulation index (cellular immunity).

TABLE 8.8

(a) Group I

	Initial	0	2	4	3/12	6/12	End
Lymphocyte Stimulation Index (mean ± SEM)							
Immunoblogulin (mean ± SEM)							
Total Ig (± SEM)							
IgG (± SEM)							
IgHA (± SEM)							
IgA (± SEM)							

(b) Group II

	Initial	0	2	4	3/12	6/12	End
Lymphocyte Stimulation Index (mean ± SEM)							
Immunoglobulin (mean ± SEM)							
Total Ig (± SEM)							
IgG (± SEM)							
IgHA (± SEM)							
IgA (± SEM)							

(c) Group III

	Initial	0	2	4	3/12	6/12	End
Lymphocyte Stimulation Index (mean - SEM)							
Immunoglobulin (mean ± SEM)							
Total Ig (± SEM)							
IgG (± SEM)							
IgHA (± SEM)							
IgA (± SEM)							

CHAPTER NINE

BUDGET AND ITS JUSTIFICATION, ETHICAL CONSIDERATIONS

9.1 Before the commencement of any research project, it is essential to estimate its cost and to establish the source of the necessary funds.

To that end, an estimate of the budget for this study is being made. Also, because of the fact that these patients are normally being seen regularly by the nephrologists and laboratory tests are being done on them for general assessment of their illness independent of this study, such tests are not charged against the study. For example, these patients are regularly being monitored for hepatitis B. Chemical tests such as BUN, creatinine, uric acid are parts of their routine medical care. Such tests are not charged to the study. Also, when any patient is ill, febrile, and has respiratory signs and symptoms, cultures would be done for etiologic diagnosis. The cultures may be only for bacteria or for both bacterial and viral agents. Such cultures should not be charged to the study nor the chest x-ray done as part of normal work up for infection.

Assessment of renal function to diagnose rejection may include renal scan, renal biopsy, etc. Such tests would be done regardless of the study. Such tests should not be charged to the study.

However, serial serologic measurements of specific CMV antibodies, all initial screenings of such tests as chest x-ray, and tests such as lymphocyte stimulation tests which are being done as part of the study should be charged to the study.

The budget does not include physician's time or laboratory consultants (who are co-investigators) time. The details of the budget as shown below take into account inflation at a rate of 7% per year.

9.2 Budget

1. Personnel: There is a need for a full-time research coordinator whose function is as described in Chapter 4 (section 4.5.2). The research coordinator is preferably an individual with B.Sc. in biologic field with research experience and/or someone with experience in research data management (and computer science background). His/her salary is set at $15,000. per year for 1½ years (total = $22,500.).

A clerical/secretary individual who has experience in technical secretarial reports and who has been involved in research activities would be needed. Her function will be as described in section 4.5.2. She probably can work part-time (4 days a week). Her salary is set at $9,000. per year (total = $13,500.).

Research assistants who preferably should be nurses or clinical fellows (who will spend at least 1½ years on a particular service) will be needed in dialysis center, transplant unit, out-patient clinic, medical ward where renal patients are looked after if admission is necessary. Arrangements could be made to admit all renal patients to a particular ward when admission is indicated. There will be five such research assistants whose functions are described in section 4.5.2. They will be required on intermittent basis. Each research assistant will receive an honorarium of $250.

2. Equipment: Stationery will be needed to the cost of $250. for the 18 months period. 300 thermometers at $1.50 each will be purchased for the patients so that they can measure their temperatures at home when indicated. Total cost for thermometers = $450. 300 diaries for daily health record at $2.00 each will cost $600. Books and files set at $80.

3. Tests: IAHA test at $10. per test. Initial screening will cost $26,000. Monthly screening of seronegative patients will cost approximately $4,000. if 60% seroconvert. Lymphocyte stimulation tests set at $30. per test. Initial cost of screening 105 patients will cost $3,150. Subsequent tests (6 tests at most per person) will cost approximately

$5,000. Chest x-ray (initial screen) at $10. per film will cost $2,700. Viral cultures at $30. per test (120 patients) will cost $3,600.

4. Data Analysis: For computer time and analysts time $1,500.

5. Miscellaneous: $500.

9.2.4 The budget can then be summarized thus:

1.	Personnel cost	$37,250.
2.	Equipment cost	1,300.
3.	Tests cost	44,450.
4.	Data analysis cost	1,500.
5.	Miscellaneous cost	500.
	TOTAL	$85,000.

With inflation, Total + 7% = 85,000. + $5,950. = $90,950.

9.3 Ethical Considerations

Patients with end-stage renal disease have serious disease. It is therefore possible for such patients to agree with their physician's demand on issues such as participation in a study. For this study, such issues are considered carefully. In the first place no more than minimum required blood will be drawn from the patients. These patients are expected to submit their blood for hepatitis B screening. The left over serum from that

routine blood test will be used to do the initial screening for CMV antibodies. The monthly blood for CMV antibody surveillence would only add 10 ml to the usual 3 monthly blood required of them. Such 10 ml will be taken when other tests (routine) are being done.

Apart from the initial chest x-ray, the only need for further x-ray will be on medical indication.

The blood for lymphocyte stimulation tests (10 ml) will be taken at the same time as when other blood tests are being done as far as possible. Utmost in the mind of the investigators will be to minimize all hazards and pain the study may expose the patients to even when the patients have agreed to participate in the study. Specifically, no patients will be billed for any assessment or tests done for the purpose of the study. Patients will be assured of freedom to withdraw from the study at any stage without prejudice to their continued care. All matters relating to the names of these patients will be dealt with confidentially. No names will be published. Data that are stored will be without name.

The object of the study, what it involves and the duration of the study will be carefully explained to each patient admitted to the study. A free informed consent will be requested from the patient before the commencement of the study.

A full protocol for the study will be drawn in advance of the study. The protocol will be presented to the Ethics Committee of St. Joseph's Hospital, Hamilton, Ontario. These measures will ensure the safety of the patients.

CHAPTER TEN

CONCLUSIONS

This thesis discusses the general issues of why it is important to study CMV among renal transplant patients more comprehensively than what is available in the literature at the present time.

The thesis also discusses the methodological issues in incidence study. It considered the problem of sensitivity and specificity of the instruments in incidence study.

The problem of adequate sample size is discussed and appropriate sample sizes are estimated for incidence, morbidity/mortaility and cellular immunity studies.

The thesis discusses the complexity of morbidity studies and designs a study to minimize some of the usual problems of morbidity studies. For example, the problem of under-reporting because the subject forgets the fact. Methodological standards in the diagnosis of morbid factors were considered as were reliability tests to validate the questionnaire.

The principles of radioactive counting are reviewed and pitfalls in lymphocyte stimulation tests discussed.

This thesis has examined a number of shortcomings in published reports. It has discussed in a major way some of these shortcomings particularly:

1. Sample size,

2. Inception cohort and knowledge of initiate state of the cohort,

3. Sensitivity, specificity and predictive value of instruments for measuring outcome of interest,

4. Minimization of both under-reporting and over-reporting in morbidity studies,

5. Sources of errors in lymphocyte stimulation (blastogenesis) studies,

The three components of the study design in this thesis can be implemented in part or as a whole. It is much cheaper to implement all the components at once.

CHAPTER ELEVEN

APPENDIX I

11.1 INFORMED CONSENT

A Study of Cytomegalovirus Among Renal Transplant and Dialysis Patients

Cytomegalovirus is the commonest virus infection a renal transplant or dialysis patient may contract. It is generally a mild disease but in patients on drugs such as azathioprine the infection can result in serious illness and/or kidney transplant rejection.

This study attempts to estimate how frequently the virus is acquired by a renal patient, the effect of the virus on body defence mechanisms and patients with transplant or dialysis do generally when they develop this virus infection as compared to those who do not develop the infection.

The study involves blood sample monthly, one or at most two x-rays, daily record of illness when they occur and questionnaire to answer once a month. The study will last 18 months.

CONSENT TO PARTICIPATE IN THE CMV STUDY

I _____ have read the information about the CMV study and it has been explained to me by Dr. _____ . I understand what the study involves. I freely volunteer to participate in the study. I understand that I can withdraw from the study at any stage without any effect on my general medical care.

Signed this _____ day of _____, 197__

_____ _____ _____

Witness Patient

APPENDIX II

11.2 Loglinear Statistical Model for Categorical Data Analysis

For the analysis of discrete data such as counts, the use of χ^2 is usually appropriate. The application of χ^2 to 2 x 2 tables is straightforward. As the table enlarged (for example, 4 x 5), the χ^2 can still be used for overall association. If this is significant, then the χ^2 can be partitioned in order to isolate the source of the association. 2 x 2, 4 x 5, 6 x 10 tables are two dimensional. There is only one plane to them. However, if the data is more than 2 dimensions the use of usual χ^2 statistics becomes difficult. The loglinear statistical model can be used to resolve such difficulties. The loglinear model is capable of isolating not only the main effects but also the interactions between the parameters.

Example: Suppose a data is 2 x 2 x 2 in form. Let X_{ijk} be the observation in i^{th} row, j^{th} column and k^{th} layer of the ratio. Let m_{ijk} be the corresponding expected values for that entry under the same model. Suppose the marginal notation is such that when adding over a variable, the subscript is replaced by a "+". Thus:

$$x_{ij+} = x_{ij1} + x_{ij2} \qquad i = 1 \quad j = 2$$

$$x_{i++} = x_{i11} + x_{i12} + x_{121} + x_{i22} \qquad i = 1, 2 = x_{il+} + x_{i2+}$$

and $x_{+++} = \sum_{i=1}^{2}\sum_{j=1}^{2}\sum_{k=1}^{2} x_{ijk} = \sum_{i=1}^{2} x_{i++}$

Similarly, $m_{ij+} = m_{ij1} + m_{ij2}$ etc.; $\hat{m}$ = expected.

Suppose $\hat{m}$ is the expected value, then for model of complete independence (all factors are indepdendent of one another).

$$\hat{m}_{ijk} = \left(\frac{X_{i++}}{N}\right)\left(\frac{X_{+j+}}{N}\right)\left(\frac{X_{++k}}{N}\right) N$$

Taking logs on both sides:

$$\log \hat{m}_{ijk} = \log X_{i++} + \log X_{+j+} + \log X_{++k} - 2 \log N$$

where N is the grand total.

Hence the expected values can be calculated. With this log function, the χ^2 can be calculated for complex tables. Practically, beyond 3 x 3 x 4, the manual calculation is cumbersome. The computer programmed to analyse data in log-linear fashion is invaluable.

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