Final Report

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Title

"Prodromal Disease: Immune Responses of the Host Macrophage System to Humoral Factors"

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I. Summary.

The following report is a composite of nine studies, each yielding information contributing toward an understanding of methods designed to detect disease during the prodromal stages. The data further point to new areas of study that might be useful in early diagnoses.

Five of the nine experiments were done in mice. Four of these involved acute infectious disease states and one involved a chronic autoimmune type disease. Of the numerous perimeters studied of the acute diseases, the uptake of ^{3}H-thymidine by peripheral blood lymphocytes appeared to yield the earliest indication of disease. This test was not useful in studying the chronic disease state.

Four of the nine studies involved application of diagnostic technics to human disease. A normal baseline for ^{3}H-thymidine incorporation by human lymphocytes was determined. A subject with severe combined immunodeficiency disease was studied. A human volunteer study was done using Influenza A live attenuated vaccine. Finally, a human volunteer study of subjects infected with Influenza A was done.

II. Introduction:

The lymphocyte appears to be the primary cell responsible for production of specific antibodies directed against all disease agents (Lawrence and Landy, 1969). To produce antibody, the lymphocyte must undergo a series of changes. It first must become stimulated, a condition that may be enhanced by contact with other cells such as macrophages. Following stimulation, the lymphocyte must synthesize new DNA, RNA, and possibly specific proteins that will lead either to division of the lymphocyte or antibody production. The uptake of ^{3}H-thymidine by lymphocytes would indicate synthesis of new DNA in these cells.

Hersh et al.(1970) first described the use of ^{3}H-thymidine uptake as an early detection device used to assay the lymphocyte response in transplant patients. They showed that this test procedure might be useful to predict early rejection in heart recipients. The present report has extended their observations to include infectious diseases, specifically mice infected with P. berghei, D. pneumoniae, E. coli B, and Hemophilus influenzae. In all cases the lymphocytes showed an increased uptake of ^{3}H-thymidine before the disease was detected by any other means.

Mice with P. berghei infection generally show a parasitemia as their first evidence of disease. The time of detection of this parasitemia depends upon the number of parasitized cells that these animals receive. As the numbers decrease, the day of onset is extended. In all cases the duration of disease following onset lasts 9 to 12 days. The ^{3}H-thymidine incorporation increases at 2 days, thus preceding any other means of detection.

The LDH isoenzymes have classically been used as indicators of early tissue destruction. In malarial infection, these enzymes were so sporadic in quantitative responses until they appeared quite unreliable as an early detection device.

Mice infected with E. coli were studied. The thymidine response was again compared with the LDH isoenzyme III response seen to rise at 18 hours. The LDH rise was consistent and significant for 18 hours but returned to non-significant values by 24 hours. The thymidine response continued to increase. The elevation in percentages of neutrophils probably reflected the animal's cellular response to rid the body of bacteria. E. coli disappeared from the blood stream by 48 hours, corresponding to the time the percentage of neutrophils returned to the normal range.

In mice infected with D. pneumoniae the infection was so fulminating that only the lymphocytes responded before death in the animals. Fever and clinical symptoms were noted 6 hours following the rise in ^{3}H-thymidine uptake by lymphocytes.

The above three studies may be found in detail in the progress report submitted previously (Criswell, 1970).

H. influenza produces pneumonia in mice characterized by an infiltrate in the lungs. This disease is fatal if high concentrations of the organisms are used. The dosage was chosen to produce disease but not death. Again the most rapidly detectible means of disease was the ^{3}H-thymidine incorporation by the lymphocytes. Detailed studies will be presented later in this report.

In each of the preceding cases the disease state was acute and had no long term implications. Malaria in mice, as stated, is fatal if contracted. To look at possible detection methods in chronic disease a serial study was undertaken to study glomerulonephritis in mice.

^{3}H-thymidine incorporation was not a useful means in studying this disease because of its chronic nature. This study was coordinated through Dr. J. M. Holland at JSC. Detailed reports are attached (Appendix I, II, III, IV, & V). Immunoglobulin deposits on the surfaces of glomeruli were detected by fluorescent antibody. The age of the animals correlated well with the severity of disease and the amount of immunoglobulin found on the surfaces of the glomeruli. The immunoglobulin accumulation appeared to give a good indication of future chronic disease in these animals.

To extend the tests developed for the mice to a human population, four studies were done. (1) A normal baseline for ^{3}H-thymidine incorporation into peripheral lymphocytes was established for 71 subjects. The mean values and statistical variations were determined. (2) An immunodeficient subject was studied extensively. (3) A human volunteer study was done using Influenza A live attenuated vaccine. (4) A human volunteer study of subjects infected with Influenza A (Hong Kong strain live and virulent) was done.

III. Methods and Materials.

All methods are recorded in Appendix VI that were used for any study in this report. Procedural approaches will be given with each individual study in the following section.

IV. Individual Studies.

A. Mice infected with P. berghei, E. coli B, and D. pneumonia: These studies have been reported previously in detail (Criswell, 1970).

B. Mice infected with Hemophilus influenza:

Procedure: A test group of normal C-57 mice were injected (I.P.) at 0 hour with H. influenza in a dosage of 1.0×10^{8} cells. Blood samples were drawn at 6 hr intervals. The lymphocytes were separated by the glass bead column method and subjected to a low concentration of ^{3}H-thymidine. Control (non-infected mice) were also drawn at each period.

Results and Discussion: The results which were assayed by scintillation counting showed no increase in ^{3}H-thymidine uptake until the 18th hour after injection from which there was a notable steady increase up to 30 hours and a decline thereafter to 48 hours after injection.

The mice showed no visible sign of illness although the increase observed in ^{3}H-thymidine by the lymphocytes points out that there was a definite effect of the organism upon the test lymphocytes. The consistently low baseline of the control mice supports this observation. Total white counts rose also indicating infection in the test animals. Again the ^{3}H-thymidine incorporation into in vivo responding lymphocytes appears to be a useful test for early detection of bacterial infection.

C. Mice with Chronic Glomerulonephritis:

Individual reports are found in detail as Appendix I, II, III, IV and V.

D. Human Studies - Normal Baselines for ^{3}H-thymidine Incorporation by Peripheral Blood Lymphocytes:

Procedure: Lymphocytes from 10 ml heparinized blood were separated by use of the Technicon Separator. 10^{6} cells were incubated with ^{3}H-thymidine for a two hour period and then the nucleoprotein incorporation rates determined.

Results: The individual values are shown in Table I. The mean value was 40 cpm with a S.D. of 11 cpm. Establishment of such baseline data will allow future comparisons with that from known infected cases.

E. Human Studies - An Immunodeficient Subject:

Procedure: Heparinized blood was obtained from an infant with severe combined immune deficiency. The individual was maintained in a germ free condition from birth to the present time. The individual was followed at monthly intervals for approximately 18 mo. The blood was spun at 2500 g for 2 min to obtain a buffy coat. The buffy coat was washed three times in PBS (pH = 7.4) and then subjected to various analyses. Wright's stained smears were examined for morphology of cells. Cells were stained with fluorescein-tagged anti-human gamma globulin (polyvalent, IgA, IgM, or IgG) for determination of B (bone marrow dependent) and T (thymic dependent) lymphocytes. Slides were made on some preparations for analysis by microspectrophotometry. Cells were fixed also for electron microscopy. Plasma was analyzed for immunoglobulin levels. Cells were cultured and stimulated with PHA, Pokeweed, and Streptolysin O to determine blastogenesis. DNA/RNA ratios were run using cytofluorometry determinations of acridine orange stained cells. All findings were compared with normal control values obtained from either children the same age or cells of known reactivity.

TABLE I

NORMAL LYMPHOCYTE STUDIES
(^{3}H-Thymidine Uptake)

Sample No.	Initials	Cts./min. (mean)
1	C.G.	44
3	P.D.	50
5	W.J.	40
6	H.L.	30
7	B.H.	23
8	R.H.	41
9	L.L.	62
10	R.P.	49
11	J.L.	51
12	N.J.	41
15	Y.	55
16	R.B.	44
18	H.J.	36
19	F.R.	52
21	A.B.	72
22	R.B.	59
23	J.W.	46
24	J.R.	47
28	C.P.	83
29	B.H.	57
47	J.W.	43
48	H.C.	42
49	L.M.	55
50	T.N.	38
51	S.C.	34
52	C.M.	37

TABLE 1 CON'T

Sample No.	Initials	Cts./min. (mean)
53	D.C.	43
54	C.H.	31
55	J.R.	33
57	J.N.	29
58	R.W.	47
59	M.S.	33
60	V.J.	32
62	R.M.	39
63	S.W.	42
64	D.G.	32
65	P.K.	30
67	J.B.	64
68	H.	32
70	J.K.	44
71	R.S.	38
72	T.S.	28
76	K.S.	39
92	J.B.	49
95	D.C.	44
97	R.K.	43
98	L.W.	56
99	M.P.	33
100	R.H.	37
104	B.M.	40
105	M.L.	45
107	E.B.	60
111	C.D.	37
113	R.V.	27
115	R.E.	55

TABLE 1 CON'T

Sample No.	Initials	Cts./min. (mean)
116	D.R.	22
117	J.T.	37
126	J.D.	36
128	R.J.	35
129	S.H.	29
130	R.R.	40
131	T.M.	53
133	P.M.	32
134	D.P.	35
138	D.P.	36
142	C.M.	43
146	M.P.	48
148	C.G.	30
150	A.F.	81
153	M.L.	24
157	R.L.	110

Results and Discussion:

1. Morphological Results.

a. Light Microscopic Findings:

At 6 weeks of age, large lymphocytes with light staining nuclei were observed in peripheral blood smears. The cells were definitely atypical with little nuclear density and very little nuclear cytoplasmic contrast. A very few small "normal" appearing lymphocytes with dark nuclei without nucleoli were seen in some fields. Of 100 lymphocytes counted 91 were 10 μ or larger, they had markedly heterogeneity of nuclear staining, 2 or more nucleoli were present, cytoplasmic vacuoles were present, and the nuclear/cytoplasmic ratio appeared small. Nine of the 100 cells were <10 μ in size with a nuclear size <9 μ, the nuclear staining was slightly heterogeneous, nucleoli of 0 or 1 were present, cytoplasmic vacuoles were absent, and the nuclear/cytoplasmic ratio was large. Following an injection with tetanus toxoid the ratio at the end of 1 month changed to 28% small lymphocytes and 72% large lymphocytes.

b. Electron Microscopic Observations:

For findings see Abstract attached in Appendix VII. Fig. 1 and 2 demonstrate the two cell types found. Figure 1 shows the depleted appearing cell while Fig. 2 shows the newer population of cell appearing at the same time as transfer factor was given.

2. B/T Cell Ratios.

On unseparated samples of buffy coat the B cell population was determined to be 96% of the circulating lymphocytes. Of this group of cells 8% contained IgA, 54%, IgG, and 48%, IgM on the cell surfaces. Technicon separated samples of blood showed only 42% lymphocytes. These ratios point to the marked thymic or T cell deficiency.

3. Nucleoprotein Analyses:

a. Microspectrophotometry:

Refer to Appendix VIII for Abstract on findings.

Figure 1. Electron micrograph of a depleted appearing cell characteristic of the majority of lymphocytes of this subject.

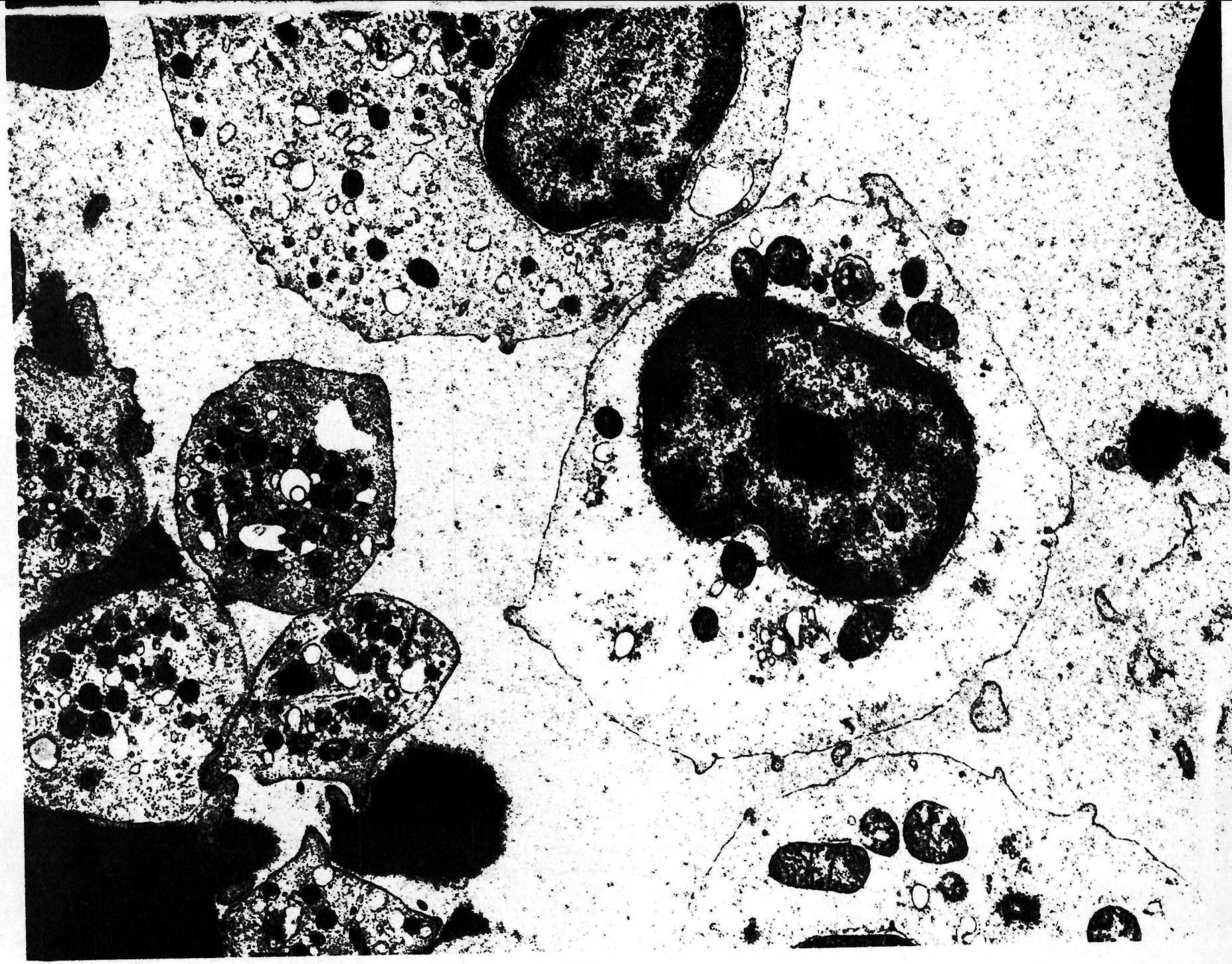
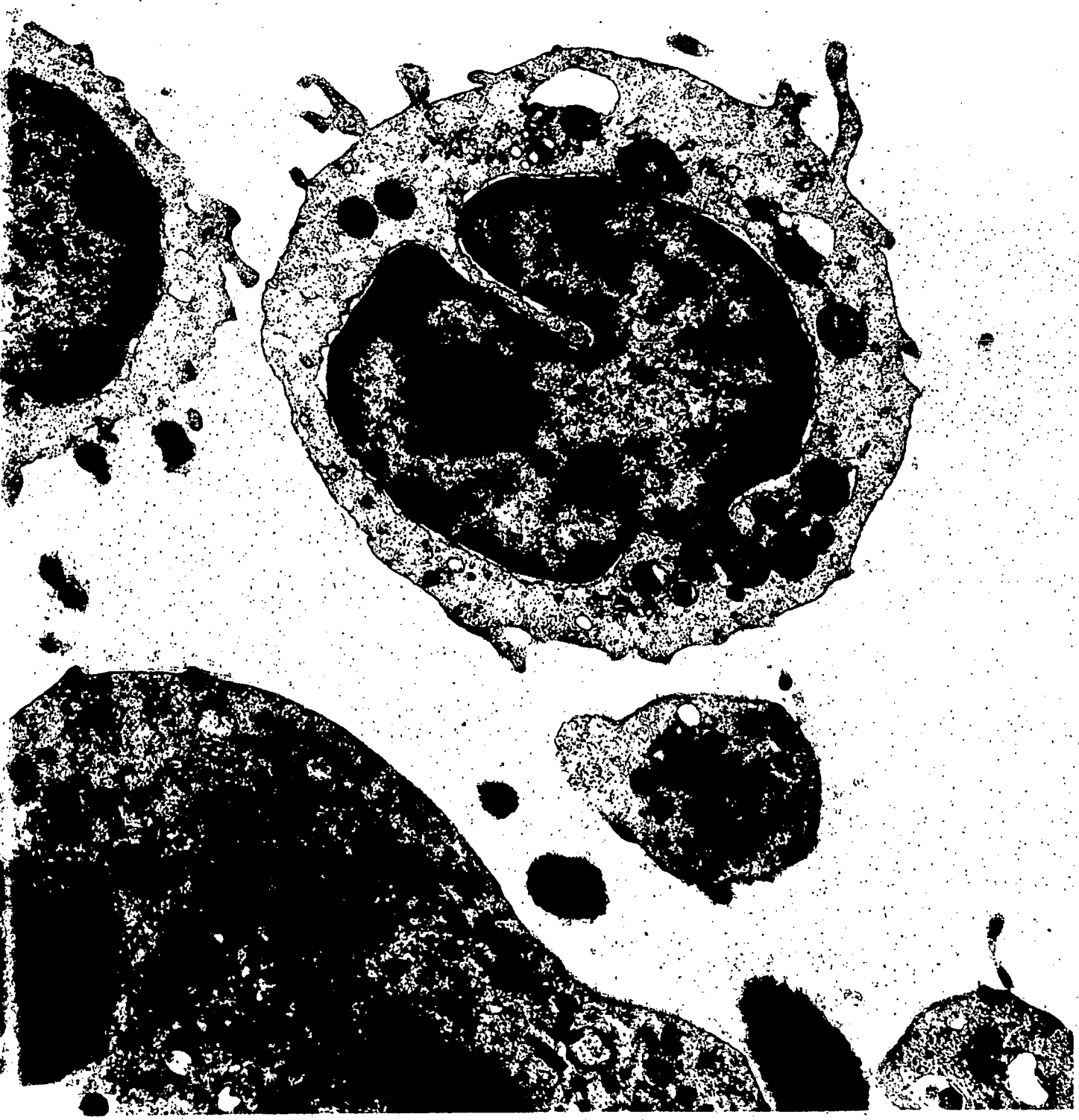


Figure 2. Electron micrograph of a more "normal" appearing lymphocyte developed following administration of transfer factor.



b. Acridine Orange binding capacity:

The lymphocytes of this subject were determined to have the same binding capacities for A.O. as normal adult lymphocytes indicating that the DNA/RNA content of these cells is normal.

4. Blastogenic Response Determined by ^{3}H-thymidine Incorporation.

No stimulation with any specific or non-specific mitogen has been recorded.

5. Plasma Immunoglobulin Levels.

Table II shows the immunoglobulin levels during the 18 mo. period. Very small quantities of Ig were produced further indicating the severe combined immunodeficiency.

F. Human Studies: A volunteer Study of Subjects Immunized with Influenza A live Attenuated Vaccine.

Procedure: Six laboratory workers who were to be immunized with the flu vaccine were studied on days -1, 0, 1, 2, 3 and 6. Vaccination was given on day 0. Peripheral blood lymphocytes were obtained by the Technicon Separator Method. 10^{6} lymphocytes were examined for ^{3}H-thymidine uptake following separation.

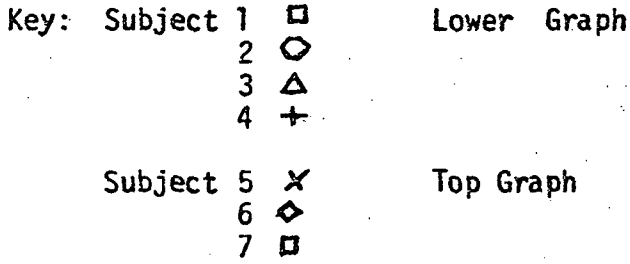
Results and Discussion: Figure 3 shows the findings. All subjects experienced a febrile reaction approximately 4-24 hrs following vaccination. None had lasting clinical symptoms. Neutralizing antibody titers were not run on the subjects prior to immunization; therefore, prior contact with the virus was not eliminated. As can be seen all subjects initially dropped or showed little significant change. The important contrast to previous data found in animals is that no initial early rise is seen in ^{3}H-thymidine incorporation in this viral infection. For this reason the following volunteer study, made possible through cooperative studies already in progress at Baylor College of Medicine, was done using a virulent viral strain. Since no initial increases were seen in ^{3}H-thymidine studies in this initial study, the lymphocytes were cultured in the subsequent study.

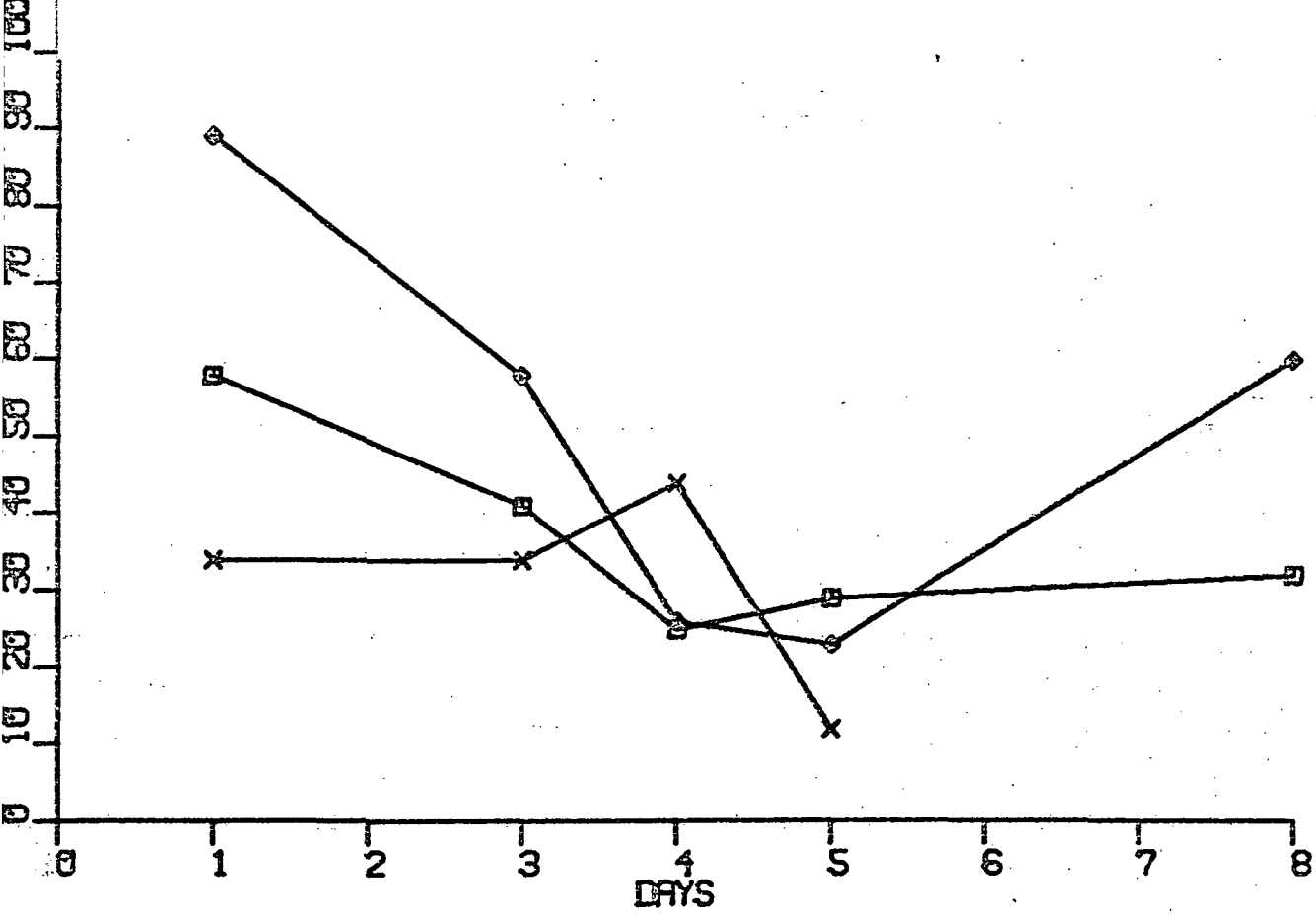
Table II

Serum Immunoglobulin Levels from an Immunodeficient Patient

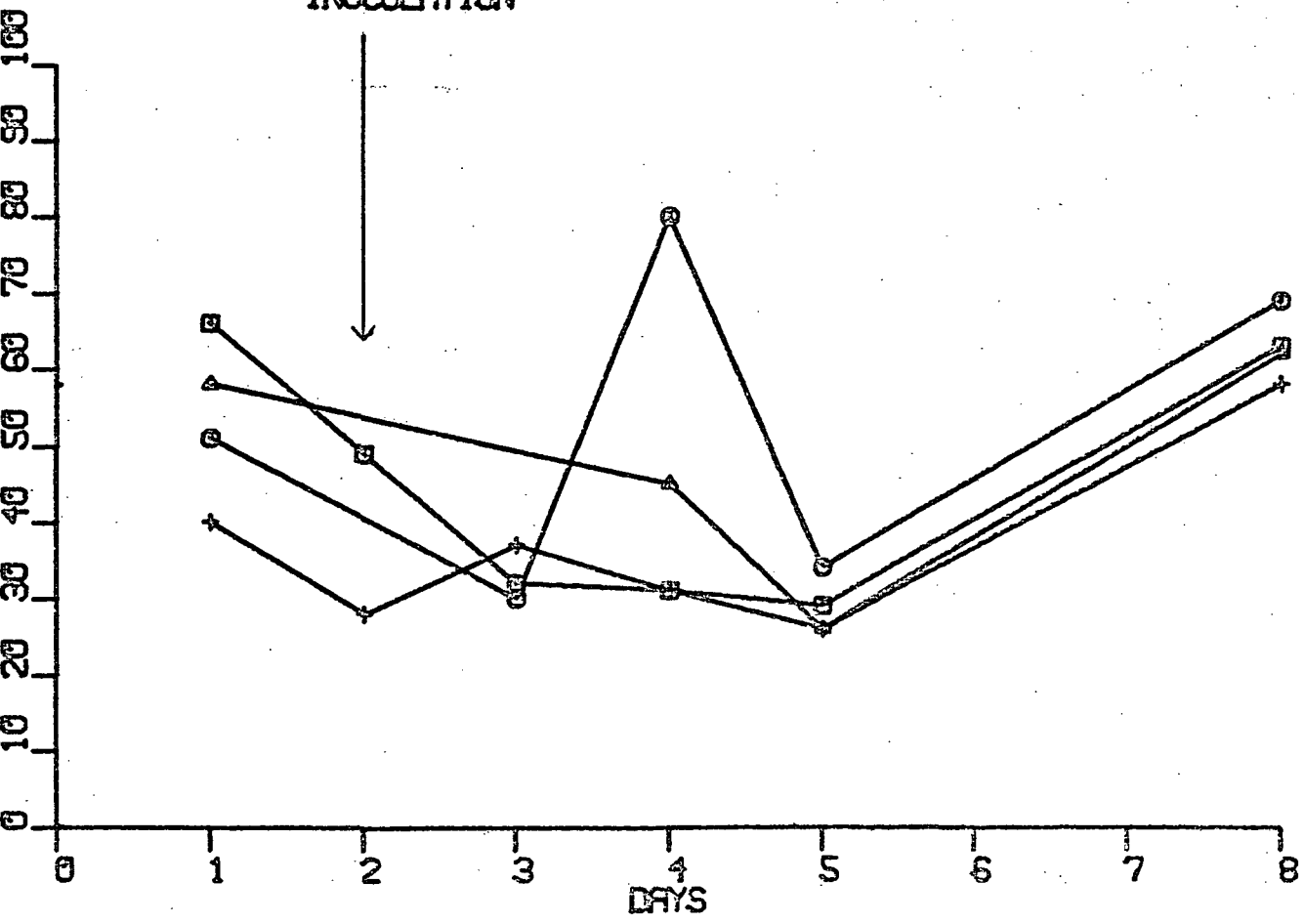
Day of Study	γ G(mg%)	γ A(mg%)	γ M(mg%)
0	292	less than 1/2	10
8	178	trace	4
22	174	trace	5.6
60	131	trace	5
104	54	trace	4.7

Figure 3. Incorporation rates of ^{3}H-thymidine into peripheral blood lymphocytes of subjects inoculated with Influenza A Vaccine.





INOCULATION



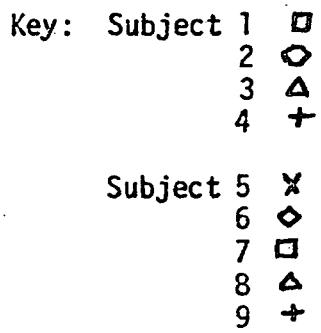
G. Human Studies - A Human Volunteer Study with Influenza A, Hong Kong Strain.

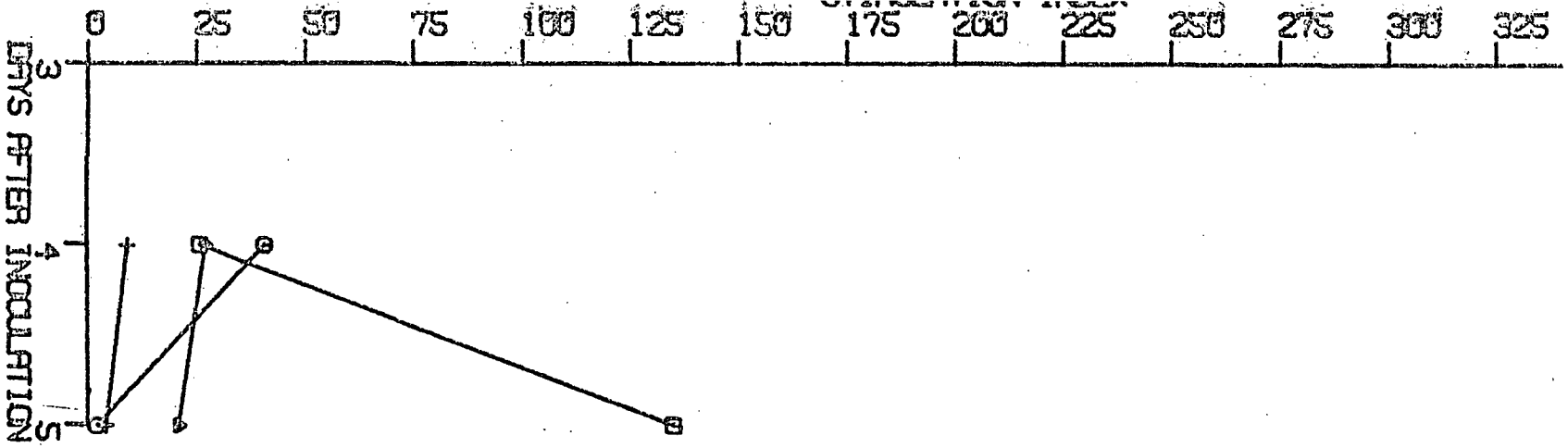
Procedure: 5 ml heparinized blood was obtained from 9 subjects infected with Influenza A by intranasal instillation. Blood samples were obtained on Day 0 before inoculation and on Days 4, 5, and 13 after inoculation. Lymphocytes were separated from the blood by use of a Technicon Lymphocyte Separator and were put into culture with PHA and Pokeweed mitogens to determine the effect of a viral infection on blastogenesis.

Results and Discussion: Figure 4 shows the results. The stimulation index (stimulated lymphocytes cpm/unstimulated lymphocytes cpm) is plotted for days 4 and 5 following inoculation for PHA cultures. All men except #1 decreased in stimulation index for PHA responses on day 5. By day 13 all subjects had again risen to values equal or greater than the day 4 stimulation index. This drop is consistent with previously published studies. It occurs about the same time as clinical symptoms appear. This change could be of value if one could find a way to examine cultured lymphocytes and detect the change prior to the 3 days required to run this test in the laboratory.

An obvious extension of this type study is to look at other properties of lymphocytes for early changes such as surface immunoglobulins, responses to the specific viral antigen itself when added to culture, and further examine the culture procedure itself for changes occurring as early as 3 or 4 hrs after adding an antigen to culture.

Figure 4. Blastogenic response of volunteers inoculated with Influenza A Hong Kong virulent strain.

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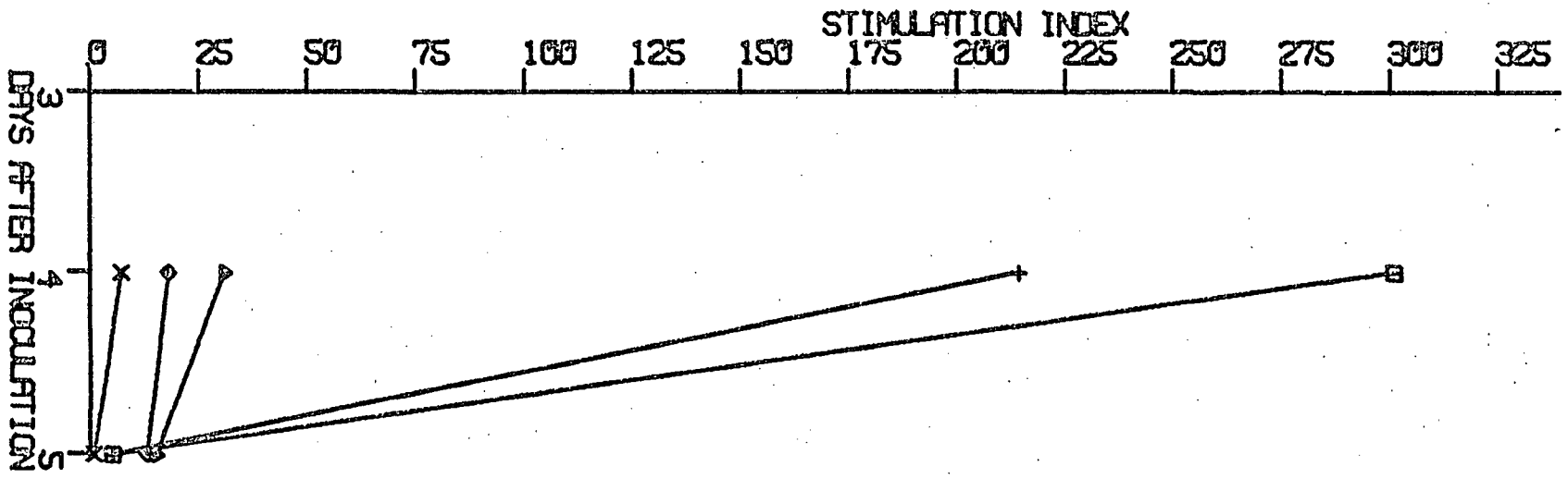
DAYS AFTER INOCULATION


STIMULATION INDEX
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Appendix

I. Progress Report: Apollo 12 Lunar Material: Effects on CD-1 Mice as Determined by Immunofluorescent Examination of Kidney, Liver, and Spleen Tissues 17 Months after Inoculation.

II. Progress Report No. 2: Effects of Lunar Material on CD-1 Mice.

III. Progress Report No. 3: Effects of Lunar Material on 5 Month Old CD-1 Mice.

IV. Progress Report No. 4: Effects of Lunar Material on 10 Month Old CD-1 Mice.

V. Final Report: Comparison of Control 2, 5, 10 & 15 month old CD-1 Mice with Mice Receiving Lunar Materials: Effects Upon a Chronic Autoimmune-Like Glomerulonephritis.

VI. Methods and Materials

VII. Ultrastructure of Lymphocytes from a Child with Severe Combined Immunodeficiency. 1973. Abstract to be published by The Texas Society for Electron Microscopy.

VIII. Changing Populations of Peripheral Blood Lymphocytes in a Gnotobiotic Child with Combined Immune Deficiency.

APPENDIX

I. Progress Report: Apollo 12 Lunar Material: Effects on CD-1 Mice as Determined by Immunofluorescent Examination of Kidney, Liver, and Spleen Tissues 17 months after Inoculation

PROGRESS REPORT

Apollo 12 Lunar Material: Effects on CD-1 Mice as Determined by Immunofluorescent Examination of Kidney, Liver, and Spleen Tissues 17 months after Inoculation

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ABSTRACT

By immunofluorescent techniques, four CD-1 mice were found to be in the moderate to severe stages of an autoimmune-like glomerulonephritis 17 months after receiving Apollo 12 lunar material. Five control animals displayed evidence of a mild to moderate disease state.

INTRODUCTION

Many strains of mice are predisposed to the development of specific diseases in their old age. Among these examples are mice that develop leukemia, tumors, and such autoimmune diseases as hemolytic anemias (1). The germ-free CD-1 Charles Rivers mice are not characterized for development of such diseases but may indeed be one of the strains that develop auto-immune type lesions.

Fresh tissues (kidney, liver, and spleens) from 18 mo. old CD-1 mice (Charles Rivers) were examined by immunofluorescent techniques. Four of the nine mice examined had received Apollo 12 lunar materials. The glomeruli of the kidneys of all the 18 mo. old animals showed a strong fluorescence when reacted with goat anti-mouse gamma globulin. The glomeruli from the older animals resemble those glomeruli of an autoimmune disease (1). Because of such small numbers, definitive conclusions could not be drawn as to the effect of the lunar materials upon the apparent disease state. One month old CD-1 mice were examined and showed no immuno-fluorescence in the glomeruli.

METHODS AND MATERIALS

Tissues from the CD-1 mice that received lunar material in the Apollo 12 quarantine study were obtained from Dr. J. M. Holland,

coordinator of the mammalian animal quarantine protocol. Frozen sections of spleen, liver, and kidney were examined for fluorescence following reaction with goat anti-mouse gamma globulin. For controls 1 month old CD-1 germ-free mice were also examined. The procedure for direct staining was described by Weir (2) with the exception of three 5 minute periods for washing slides. Photographs were taken on a Leitz Orthomat photomicroscope with the aid of the Leitz automatic camera. Exciter filters UG 1 and BG 12 were used.

RESULTS

All the 18 mo. old mice show signs of kidney disease. The glomeruli of these animals fluoresced brightly following reaction with anti-mouse gamma globulin (Fig. 1 and 2). The kidneys examined show various stages of illness as indicated by the size of the glomeruli and presence of an amorphous substance. Fig. 1 shows the condition of mouse No. 642 which did not receive lunar material. A fluorescent glomerulous that has not enlarged appreciably in size and is intact in appearance is seen. The tubular areas have essentially no materials within their lumens indicating a relatively intact kidney. In contrast, Fig. 2 shows one of the most progressive

Fig. 1. Glomeruli from an 18 month old CD-1 mouse No. 642 that had received no lunar material

NASA Photo No. S-71-41420

Fig. 2. Glomeruli from an 18 month old CD-1 mouse No. 562 that had received lunar material

NASA Photo No. S-71-41421

disease states seen in mouse No. 562 which received Apollo 12 lunar material. Thickened basement membranes of greatly enlarged glomeruli filled with an amorphous material are seen. The tubules contain gamma globulins, indicating loss in normal kidney function. Fig. 3 shows tissue from CD 1 germ-free 4 month old animals. No fluorescence is found.

Three stages of disease are apparent and are described in Table 1.

TABLE 1

Conditions of Glomeruli Examined by Immunofluorescence Techniques

	States of Disease		
	Category I	Category II	Category III
Animals receiving no lunar rock	642 634	639 633 636	
Animals receiving lunar rock		531 532*	565 562

*532 had a hemolytic anemia in addition to the glomerulonephritis.

The animals classified as Category I, least effected, had fluorescent labelled glomeruli that were intact with no enlargement, no appreciable thickening of the basement membranes and minimal if any amorphous material present. Category II, the moderately effected

Fig. 3. Kidney tissue from 4 month old germ free CD-1 mice

NASA Photo No. S-71-41419

group, had slightly enlarged, but still intact, glomeruli. Some amorphous infiltrate was apparent. The two mice in Category III were the most severely effected. They displayed large increases in size of the glomeruli, some of which were no longer intact. Convolutions were essentially gone, and massive amounts of the amorphous infiltrate were present. In these two animals, the infiltrate, along with fluorescent foci of immunoglobulin producing cells, was present in the spleen and liver tissues as well as the kidney.

DISCUSSION

The present study indicates that an autoimmune-like glomerulonephritis was present in both 18 mo. old control and test animals of the Apollo 12 mice. The severity of the disease varied among the different animals; however, since only 9 animals were examined, definitive conclusions concerning the lunar material's effect upon the underlying disease could not be determined. Animals receiving the added irritation of the lunar materials appeared to progress more rapidly in their disease states than the controls. However, since no baseline studies exist for purposes of comparison and no statistical variations are available, any conclusions can be only speculation.

The effects seen in these animals need to be characterized further using more defined systems with larger numbers of animals for statistical analyses. The effects of lunar material upon autoimmune phenomenon or upon chronic disease states have not been defined. Furthermore, comparisons should also be made for effects seen when simulated lunar materials are used as an added control for the lunar materials injected into animals.

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APPENDIX

II. Progress Report No. 2: Effects of Lunar Material on CD-1 Mice

PROGRESS REPORT NO. 2

EFFECTS OF LUNAR MATERIAL ON CD-1 MICE

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ABSTRACT

Kidney tissues from mice that were 15 mo old and had received no previous injections were examined by immunofluorescent techniques. 87% of these uninjected animals showed positive immunofluorescence or immunoglobulin on their glomeruli. None of the 57 animals examined possessed disease states as severe as previously seen in the 17 mo Apollo 12 lunar injected mice.

INTRODUCTION

Previous studies (Report # 1) indicated that CD-1 Charles Rivers mice examined 17 months after receiving injections of lunar material might display enhanced severity of the glomerulonephritis characteristic of aging mice. Since the CD-1 mice had not been characterized for such lesions, a series of control animals are being studied to establish a baseline for them as a continuing part of the lunar bio-characterization program at MSC. The nature or actual cause of the glomerulonephritis, however, is unknown. It is most probably caused by viral agents within the basement membranes during the life of the animal and similar to the diseases described by Oldstone and Dixon (2).

In this report the examination of a control group of CD-1 Charles Rivers 15 mo old mice are reported. Of the 57 mice examined 87% had indications of disease by immunofluorescence techniques.

METHODS AND MATERIALS

Tissues from the 57 control animals were obtained from Dr. J. M. Holland, coordinator of the mammalian animal quarantine protocol. Frozen sections of the kidneys were examined for fluorescence following reaction with goat anti-mouse gamma globulin. Selected positive tissues were

further examined for fluorescence with goat anti-mouse IgG_1, IgG_2, IgA, and IgM. Exact procedures and photography methods were previously described (1).

RESULTS

Tabulated results are shown in Tables 1 and 2. The individual animals are grouped and are compared to those animals examined and reported in Report 1 (1). The criteria for each category are defined in a note to Table 1. Categories I, II, and III of Report 1 correspond to categories 2, 3, and 4 respectively of the present report. The new categories 0, and 1 represent less severe disease states that were not seen in the 17 mo CD-1 animals described in Report 1. As can be seen in Table 2, 56% of the animals were in very early stages of glomerulonephritis (category 1). 31% were in categories 2 and 3. No animals examined were in the final stages of glomerulonephritis and subsequent total kidney failure (category 4).

The germ free males, germ free females, and conventional males all yielded similar data. The conventional females (CF) represented a divergent group with significantly more severe stages of disease. 43% of the CF were in category 2 while the other three groups had 15-19% in the same category.

IgM only was found on the glomeruli as the bound immunoglobulin.

TABLE 1

oup signation	Animal Numbers	Categories				
		0	1	2	3	4
mo Germ ee Males	045, 048, 050	3				
	041, 042, 043, 044, 049, 051, 052, 062		8			
	046			1		
	047				1	
	None					0
mo Germ ee Females	058, 059	2				
	061, 063, 064, 065, 066, 067, 068		7			
	060			1		
	053, 057				2	
	None					0
mo nventional les	079	1				
	073, 074, 077, 078, 080, 081, 082, 083, 085, 086, 087, 088		12			
	075, 084			2		
	076				1	
	None					0
mo nventional males	095	1				
	089, 097, 101, 102, 103		5			
	091, 092, 093, 094, 095 099, 104			7		
	090, 098, 100				3	
	None					0

Note to Table 1.

Category 0, no fluorescence

Category 1, any small degree of fluorescence in any glomerulous, otherwise essentially normal appearance

Category 2, all glomeruli fluorescing, no enlargement noted

Category 3, all glomeruli fluorescing, 50% beginning to enlarge but convolutions still intact

Category 4, all glomeruli fluorescing and enlarged, convolutions and normal structure are missing.

TABLE 2

Group Designation	Percentage (Actual No.) of Animals in Categories				
	0	1	2	3	4
Controls:					
A. Present Study					
1. Germ Free Females, 15 mo	17(2)	58(7)	8(1)	17(2)	0
2. Germ Free Males, 15 mo	23(3)	62(8)	8(1)	8(1)	0
3. Conventional Females 15 mo	6(1)	31(5)	44(7)	19(3)	0
4. Conventional Males 15 mo	6(1)	75(12)	13(2)	'6(1)	0
B. Data from Report 1. Conventional Males & Females	0	0	40(2)	60(3)	0
Injected: Data from Report 1. for Apollo 12 Lunar Material Injections	0	0	0	50(2)	50(2)

DISCUSSION

The major conclusion that may be drawn from the present data is that the degree of involvement in the 15 mo old control animals of this study is not as advanced as that seen in the 17 mo injected animals in the previous Report 1(1). 12% of the 15 mo controls show category 3 involvement, and none were in category 4. 20 month old control animals will be examined at a later date to determine their status.

The need for a more controlled disease state to study how lunar materials may be interacting in the biological system is becoming more pressing. The scope of a project to define the glomerulonephritis seen in the CD-1 Charles Rivers mice would require more time, technical assistance, and equipment than is presently available. Oldstone and Dixon (2) have only recently, after many years effort, succeeded in describing glomerulonephritis due to IgG and virus attachment to the glomerular basement membranes. Only one other group of investigators (3) have described IgM attachment as is seen by us. These investigators are still in the process of describing the disease and causative agents.

By examining the effect of lunar and lunar simulated materials in an induced controlled disease state, one could

determine the stages in the biological processes that might be involved and further determine if the lunar and simulated lunar materials do indeed act the same. If indeed they do act similarly then one could try various combinations of individual elements from the similated materials to determine which ones might be the most important in influencing biological systems under stress from ensueing disease.

References

1. Criswell, B. Sue. 1971. Apollo 12 Lunar Material: Effects on CD-1 mice as determined by immunofluorescent examination of kidney, liver, and spleen tissues 17 months after inoculation. Progress report submitted to Dr. M. Holland and Dr. W.W. Kemmerer, Jr., Preventive Medicine Division, NASA-MSC.
2. Oldstone, M.B.A., and F. J. Dixon. 1971. Virus-Antiviral Antibody Complexes. Progress in Immunology (Academic Press, New York) pp. 763-777.
3. Hirsch, M., A. Allison, and J. Harvey. 1969 Nature 223:739.

APPENDIX

III. Progress Report No. 3: Effects of Lunar Material on 5 month old CD-1 Mice

Progress Report No. 3

(January 24, 1972)

Effects of Lunar Material on 5 month old CD-1 Mice

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ABSTRACT

Kidney tissues from two groups of mice were examined by immunofluorescent techniques. One group of mice was 6 mo. old and had been injected with either lunar material or terresterial material 5 mo. previously. A second group of mice were 5 mo. old and had received no prior injections. 60% of the 5 mo. old animals already had bound immunoglobulin on their glomeruli.

Introduction:

As a continuing part of the lunar bio-characterization program, 5 mo. old control and lunar inoculated animals were examined for kidney lesions evidenced by immunofluorescence.

Methods & Materials:

Procedures have been given previously (Reports 1 & 2).

Results & Discussion:

Tabulated results are shown in Tables 1 & 2. The individual animals are grouped and results summarized in Table 3. The criteria for each category was given previously in Report 2 (2). As can be seen in Table 3, 60-63% of all 5 mo. old CD-1 mice studied were in the early stages of glomerulonephritis (Categories 1 & 2). One animal was in more advanced stages (Category 3); however, this animal (#125) did not possess the same type of kidney disease as all the others. The predominant immunoglobulin on this kidney was IgG with some IgM. On all other animals IgM with trace amounts of IgA were found but no IgG. There appeared to be no significant difference between the control animals and the injected ones indicating that the lunar material or simulated lunar material seemed to have no disease enhancing capacity. There was also no difference between

the lunar injected animals and the terresterial group.

One significant difference did appear among the control animals. Control conventional females displayed enhanced stages of disease compared with the conventional males, germ free males, and germ free females.

References:

1. Criswell, B.S. 1971. Apollo 12 Lunar Material: Effects on CD-1 mice as determined by immunofluorescent examination of kidney, liver and spleen tissues 17 months after inoculation. (Progress report submitted to Dr. H. Holland and Dr. W. W. Kemmerer, Jr., Preventive Medicine Division, NASA-MSC.)

2. Criswell, B.S. 1972. Progress Report No. 2: Effects of Lunar Material on CD-1 Mice. (Progress report submitted to Dr. H. Holland and Dr. W. W. Kemmerer, Jr., Preventive Medicine Division, NASA - MSC.)

TABLE 1

Group Designation	Animal Numbers	Catagories 0	1	2	3	4
Conventional Males 5 mo. CD-1	138, 139, 140, 144, 147, 150, 152	7				
	137, 141, 142, 143, 145, 146, 149, 151		8			
	148			1		
	None				0	
	None					0
Conventional Females 5 mo. CD-1	None	0				
	153, 154, 156, 159, 162, 163, 164, 165, 166		9			
	155, 157, 158, 160, 161, 167, 168			7		
	None				0	
	None					0
Germ free Males 5 mo. CD-1	107, 109, 111, 112, 113, 116, 117, 118, 119	9				
	105, 106, 108, 110, 114, 115		6			
	None			0		
	None				0	
	None					0
Germ free Females 5 mo. CD-1	126, 128, 129, 132, 133, 134	6				
	121, 122, 124, 127, 130, 131, 135, 136		8			
	123			1		
	125				1	
	None					0

TABLE 2

Group Designation	Animal Numbers	Catagories 0	1	2	3	4
Conventional	001, 002, 003, 006, 009, 010	6				
CD-1 5 mo.	004, 005, 007, 008,		4			
Terr. Inj. Males	None			0		
	None				0	
	None					0
Conventional	018, 019	2				
CD-1 5 mo.	012, 014, 015, 016, 017, 020		6			
Terr. Inj. Females	011, 013			2		
	None				0	
	None					0
Conventional	021, 024, 025, 026, 027, 028	6				
CD-1 5 mo.	022, 023, 029, 030		4			
Lunar Inj. Males	None			0		
	None				0	
	None					0
Conventional	034, 035	2				
CD-1 5 mo.	031, 032, 033, 036, 037, 038, 040		7			
Lunar Inj. Females	039			1		
	None				0	
	None					0

TABLE 3

Group Designation	Percentage (Actual No.) of Animals in Catagories 0	1	2	3	4
Controls:					
1. Conventional Males	44(7)	50(8)	6(1)	0	0
2. Conventional Females	0	56(9)	44(7)	0	0
3. Germ Free Males	60(9)	40(6)	0	0	0
4. Germ Free Females	37(6)	50(8)	6(1)	6(1)	0
Injected:					
1. Conventional Males - Terresterial Material	60(6)	40(4)	0	0	0
2. Conventional Females - Terresterial Material	20(2)	60(6)	20(2)	0	0
3. Conventional Males Lunar Material	60 (6)	40 (4)	0	0	0
4. Conventional Females - Lunar Material	20 (2)	70 (7)	10 (1)	0	0
Cumulative Totals:					
1. 5 mo. Controls	35(22)	49(31)	14(9)	2(1)	0
2. 5 mo. Injected Animals	40(16)	53(21)	7(3)	0	0

APPENDIX

IV. Progress Report No. 4: Effects of Lunar Material on 10 month old CD-1 Mice

Progress Report No. 4

(March 6, 1972)

Effects of Lunar Material on 10 Month Old CD-1 Mice

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Abstract

Kidney tissues from 10 mo. old CD-1 mice that were either germ free or conventionally housed were examined by immunofluorescent techniques. 72% of the 10 mo. old animals had bound immunoglobulin on their glomeruli.

Introduction:

As a continuing part of the lunar bio-characterization program, 10 mo. old CD-1 mice both germ free and conventional were examined for kidney lesions evidenced by immunofluorescence.

Methods and Materials:

Procedures have been given previously (Reports 1&2).

Results and Discussion:

Tabulated results are shown in Tables 1 & 2. The criteria for each category was given previously in Report 2 (2). As can be seen in Table 2, 72% of all 5 mo. old CD-1 mice studied were in the early stages of glomerulonephritis (Categories 1 & 2). One animal (#217) was in more advanced stages (Category 3). Staining with Anti IgA, IgM, IgG_1, and IgG_2 showed that the predominant immunoglobulin present was IgM; however, small amounts of the other immunoglobulins were also detectable.

There appeared to be a significant difference between the conventional females and the other groups. The germ free males appeared least involved. Again, as seen previously, the females as a group were in more advanced stages than the males.

References:

1. Criswell, B.S. 1971. Apollo 12 Lunar Material: Effects on CD-1 mice as determined by immunofluorescent examination of kidney, liver and spleen tissues 17 months after inoculation. (Progress report submitted to Dr. H. Holland and Dr. W. W. Kemmerer, Jr., Preventive Medicine Division, NASA-MSC.)

2. Criswell, B.S. 1972. Progress Report No. 2: Effects of Lunar Material on CD-1 Mice. (Progress report submitted to Dr. H. Holland and Dr. W. W. Kemmerer, Jr., Preventive Medicine Division, NASA-MSC.)

Table 1

Group Designation	Animal Numbers	Categories 0	1+	2+	3+	4+
10 Mo. GF MALES	169, 171, 172, 173, 174, 175 176, 177, 178, 179, 181	11				
	170, 180, 182, 183		4			
	184			1		
	None				0	
	None					0
10 Mo. GF FEMALES	192, 197, 198, 200	4				
	185, 186, 187, 188, 191, 193, 194, 195, 196, 199		10			
	189, 190			2		
	None				0	
	None					0
10 Mo. CONV. MALES	210, 211, 212	3				
	201, 202, 203, 204, 205, 206, 207, 208, 209, 214, 216		11			
	213, 215			2		
	None				0	
	None					0
10 Mo. CONV. FEMALES	None	0				
	219, 222, 223, 224, 225, 226, 227, 229, 232		9			
	218, 220, 221, 228, 230, 231			6		
	217				1	
	None					0

Table 2

Group Designation	Percentage (Actual No.) of Animals in Categories 0	1	2	3	4
Germ Free Males	69(11)	25(4)	6(1)	0	0
Germ Free Females	25(4)	63(10)	12(2)	0	0
Conventional Males	19(3)	69(11)	12(2)	0	0
Conventional Females	0	56(9)	38(6)	6(1)	0
Cumulative Total	28(18)	53(34)	17(11)	2(1)	0

APPENDIX

V. Final Report: Comparison of Control 2, 5, 10, & 15 month old CD-1 Mice with Mice Receiving Lunar Materials: Effects Upon a Chronic Autoimmune-Like Glomerulonephritis.

Final Report

(April 15, 1973)

Comparison of Control 2, 5, 10, and 15 Mo. Old CD-1 Mice with Mice Receiving Lunar Materials: Effects Upon a Chronic Autoimmune-like Glomerulonephritis.

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Abstract

Of 23 CD-1 2 mo. old mice, 4 had evidence of immunoglobulin on the surfaces of their kidneys, indicating a very early beginning of an autoimmune-like disease in these animals. As the animals aged, the kidneys became progressively effected.

Lunar materials or terresterial simulated lunar materials injected I.P. did not alter the chronic disease course.

INTRODUCTION:

As a continuing part of the lunar biocharacterization program, 2 mo. old CD-1 mice, both germ free and conventional, were examined for kidney lesions evidenced by immunofluorescence. This report concludes the control study for the biocharacterization program. CD-1 mice receiving either terresterial simulated lunar materials or lunar materials and allowed to age to 10 months and 15 months, respectively, were examined for their response to this chronic disease state.

METHODS AND MATERIALS:

Procedures have been given previously (Reports 1-4).

RESULTS:

Tabulated results are shown in Table I for 2 mo. old CD-1 mice. The criteria for each category has been given previously in Report 2 (2). In addition the categories have been evaluated photometrically by use of a Leitz MPV. Category 0 photometrically measured between 100-450 OD; Category 1 = 400 - 600; Category 2 = 500 - 1000; Category 3 = 1000 - 2500; Category 4 = 2000 - 4500. The photometric readings measured only intensity of fluorescence. The categories were established to include other perimeters. Therefore, overlapping of numerical values occurred.

Additional control studies were done to show the specificity of the immunoglobulins used. The antisera were tested electrophoretically to determine purity and were blocked with unlabelled antisera [i.e., anti-IgM unlabelled blocked Anti-IgM (fluorescein tagged) from binding to the glomerulous]. All antisera used were established pure by both methods.

Results of injecting 2 mo. old mice with either lunar or terresterial simulated lunar materials and then examination at 10, and 15 mo. of age are shown in Tables II and III. In Table II, males at 10 mo. injected with lunar material had more advanced disease than males injected with terresterial material. On the other hand, females with lunar material evidenced less disease than females with terresterial materials. Upon comparison with control animals in Table IV no statistical differences were observed. Too few germ free females survived until 15 mo. to analyze. However, the males at 15 mo. (Table III) did not differ regardless of what they had received.

DISCUSSION:

The previous three studies and this one point to the fact that the CD-1 strain of mice develop normally an accumulation of immunoglobulin on their glomeruli basement membranes resembling the disease of the NZB or B/W hybrid mice (Burnet, 1972). The actual etiology

of this condition remains a subject of extensive debate. The females appear to be affected sooner than the males during the course of their lives. The germ free animals are slower to contract the disease than the conventionally housed animals.

Those animals that received lunar materials did not differ from those animals receiving terresterial simulated lunar material in severity of the disease. The injected animals tended to fall lower than the non-injected controls at 5 mo. of age. However, by 10 mo. all groups appeared equal in distribution of the disease. The injected materials appeared to have little lasting effects upon the course of this naturally occurring chronic disease state.

References

1. Criswell, B.S. 1971. Apollo 12 Lunar Material: Effects on CD-1 Mice as determined by immunofluorescent examination of kidney, liver, and spleen tissues 17 months after inoculation. (Progress report submitted to Dr. H. Holland and Dr. W. W. Kemmerer, Jr., Preventive Medicine Division, NASA-MSC).

2. Criswell, B.S. 1972. Progress Report No. 2: Effects of Lunar Material on CD-1 Mice. (Progress report submitted to Dr. H. Holland and Dr. W. W. Kemmerer, Jr., Preventive Medicine Division, NASA-MSC).

3. Criswell, B.S. 1972. Progress Report No. 4: Effects of Lunar Material on 10 Month Old CD-1 Mice. (Progress report submitted to Drs. Holland and Kemmerer,Jr., Preventive Medicine, NASA-MSC).

4. Burnet, Sir Macfarlane. 1972. Auto-immunity and Auto-immune Disease. F. A. Davis Co. (Philadelphia) p. 243.

Table I

Group Designation	Animal Numbers	Categories 0	1+	2+	3+	4+
Germfree 2 mo. Males	313, 314, 315, 316, 317, 318, 320	7				
	319		1			
Germfree 2 mo. Females	322, 323, 324, 327, 332, 333, 334, 335	8				
	336		1			
Conventional 2 mo. Males	382, 383, 385	3				
	384		1			
Conventional 2 mo. Females	338	1				
	337		1			

Table II

Apollo 15 - 10 mo. CD-1

		0	1+	2+	3+	4+
Lunar Injected:						
Males, 10 mo. (Conventional)	233, 239, 240	(3)30%				
	234, 235, 236 237, 238, 241		(6)60%			
	242			(1)10%		
					0	0
Females, 10 mo. (Conventional)	252	(1)12.5%				
	244, 246, 247, 248, 250		(6)75%			
	251			(1)12.5%		
					0	0
Terr. Injected:						
Males, 10 mo. (Conventional)	254, 255, 257, 258, 260, 261, 262	(7)70%				
	253, 256, 259		(3)30%			
				0	0	0
Females, 10 mo. (conventional)	270	(1)10%				
	263, 265, 268, 269		(4)40%			
	264, 267, 272			(3)30%		
	266, 271				(2)20%	
						0

Table III

Apollo 15 - 15 mo. CD-1

		0	1+	2+	3+	4+
Lunar Injected:						
Males, 15 mo. (Germfree)	386	(1)12.5%				
	382, 383, 384, 387		(4)50%			
	385, 388			(2)25%		
	389				(1)12.5%	
	None					(0)
Females, 10 mo. (Germfree)	394	(1)17%				
	393, 395		(2)33%			
	390, 392			(2)33%		
	391				(1)17%	
	None					(0)
Terr. Injected:						
Males, 15 mo. (Germfree)	396	(1)17%				
	398, 400, 401		(3)50%			
	397, 399			(2)33%		
	None				(0)	
	None					(0)
Females, 15 mo. (Germfree)	None	(0)				
	402		(1)100%			
	None			(0)		
	None				(0)	
	None					(0)

Table IV

Summary Table of Comparative Data

oup signation	Percentage (Actual No.) of Animals in Categories				
	0	1	2	3	4
ntrols:					
2 mo. Conv. Males	66(3)	33(1)	0	0	0
2 mo. Conv. Females	50(1)	50(1)	0	0	0
2 mo. Germfree Males	88(7)	12(1)	0	0	0
2 mo. Germfree Females	89(8)	11(1)	0	0	0
5 mo. Conv. Males	44(7)	50(8)	6(1)	0	0
5 mo. Conv. Females	0	56(9)	44(7)	0	0
5 mo. Germfree Males	60(9)	40(6)	0	0	0
5 mo. Germfree Females	31(5)	56(9)	6(1)	6(1)	0
10 mo. Conv. Males	19(3)	69(11)	12(2)	0	0
10 mo. Conv. Females	0	56(9)	38(6)	6(1)	0
10 mo. Germfree Males	69(11)	25(4)	6(1)	0	0
10 mo. Germfree Females	25(4)	63(10)	12(2)	0	0
15 mo. Conv. Males	6(1)	75(12)	13(2)	6(1)	0
15 mo. Conv. Females	6(1)	31(5)	44(7)	19(3)	0
15 mo. Germfree Males	23(3)	62(8)	8(1)	8(1)	0
15 mo. Germfree Females	17(2)	58(7)	8(1)	17(2)	0
jected:					
5 mo. Conv. Males: Terresterial Mat.	60(6)	40(4)	0	0	0
5 mo. Conv. Females: Terr. Material	20(2)	60(6)	20(2)	0	0
5 mo. Conv. Males: Lunar Material	60(6)	40(4)	0	0	0
5 mo. Conv. Females: Lunar Material	20(2)	70(7)	10(1)	0	0
10 mo. Conv. Males: Terr. Material	70(7)	30(3)	0	0	0
10 mo. Conv. Females: Terr. Material	10(1)	40(4)	30(3)	20(2)	0
10 mo. Conv. Males: Lunar Material	30(3)	60(6)	10(1)	0	0
10 mo. Conv. Females: Lunar Material	13(1)	75(6)	13(1)	0	0
15 mo. Conv. Males: Terr. Material	17(1)	50(3)	33(2)	0	0
15 mo. Conv. Females: Terr. Material	0	100(1)	0	0	0
15 mo. Conv. Males: Lunar Material	13(1)	50(4)	25(2)	13(1)	0
15 mo. Conv. Females: Lunar Material	17(1)	33(2)	33(2)	17(1)	0

Table IV (continued)

	0	1	2	3	4
Cumulative Totals:					
5 mo. Controls	33(21)	51(32)	14(9)	16(1)	0
5 mo. Terresterial Material	40(8)	50(10)	10(2)	0	0
5 mo. Lunar Injected	40(8)	55(11)	5(1)	0	0
10 mo. Controls	28(18)	53(34)	17(11)	2(1)	0
10 mo. Terresterial Injected	40(8)	35(7)	15(3)	10(2)	0
10 mo. Lunar Injected	22(4)	67(12)	11(2)	0	0
15 mo. Controls	12(7)	56(32)	19(11)	12(7)	0
15 mo. Terresterial Injected	14(1)	57(4)	29(2)	0	0
15 mo. Lunar Injected	14(2)	43(6)	29(4)	14(2)	0

APPENDIX

VI. Methods and Materials

LYMPHOCYTE SEPARATION

using Techincon Separator

If lymphocytes are to be cultured later, the blood sample should be collected in a sterile, heparinized tube and all supplies used hereafter in the separation must be sterile.

If lymphocytes are not to be cultured, non-sterile supplies can be used.

1. Remove separating reagent from refrigerator and place in a 37°C incubator or H_2O bath for approx. 1 hr. or allow to sit at room temp. for 2 hrs.
2. Collect blood in a 15 ml. vacutainer tube containing 3 mls. Na Heparin. Make these tubes up in advance to keep on hand. If another size vacutainer is used, use 4 ml. Na Heparin/20 ml. blood.
3. Type the blood with ABO typing serum.
4. If more than 5 mls. of blood are collected, the sample may be spun down at setting 3/4 on ICC centrifuge for 15-20 mins. Take off most of the serum and save. Remove the buffy coat generously, taking some RBC's and the remaining serum that was left and place in a vial.
5. Take separating reagent and shake well to mix the iron filings. Using a 50cc. B-D syringe and a 20g. needle, withdraw reagent in the amount of 4X the volume of blood.
   Ex.: For 5 mls. of blood, use 20 mls. reagent.
6. Withdraw into the syringe approx. 1 ml. of typing antisera according to the person's blood type.
7. If the buffy coat is being used, mix it well with a pasteur pipette. Draw the blood into the syringe containing the reagent and antisera.
8. Draw an airspace into syringe equal to the volume of the mixture and cap the needle with a vinyl cap.
9. Mix by inverting several times.
10. Place the syringe on Technicon rotator which is kept in a 37°C incubator. Allow to rotate for 30 mins.
11. Remove syringe from rotator, remove the airspace and tap out any air bubbles present, and replace the needle with a clean one.
12. Allow syringe to sit in the rack (also in incubator) for 30 mins.
13. Take the sample through the Technicon Separator. If the sample is to be kept sterile, wipe the tubing and the top of the collection tube and its holder with alcohol and allow to dry before inserting needles.
14. Spin the collected sample in the tube for 10 mins. at speed 3/4 on ICC centrifuge.

15. Remove the supernatant and discard.

16. Using a long pasteur pipette, extract the pellet, rinsing with MEM, into a fisher tube.

17. Spin at 2500g for 2 mins. or 1500g for 3 mins. Remove Supernatant.

18. Rinse pellet 2X with MEM as above and place in 1 ml. MEM.

19. Mix well and count on Hemacytometer. Count the 5 small squares.

20 If the sample has too many REC's to count, use diluting pipette. Count the 4 large squares.

LYMPHOCYTE CULTURE TECHNIQUE

Blood Collection

1. 10-20 ml. blood is drawn and placed into a 50 ml. sterile erhlenmeyer flask containing sterile acid washed glass beads (3 beads/1 ml. blood; beads are 3mm in diameter).

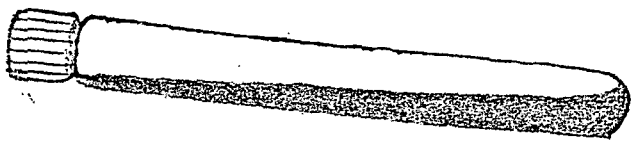
2. Swirl flask for 10-15 min. until beads can no longer be heard rattling in flask (but do not swirl so hard as to induce frothing of blood).

Cell Separation

1. In sterile culture tubes add 1 part Sterile (autoclaved) 4% dextran in saline for every 9 parts of blood.

2. Transfer the defibrinated blood to the dextran culture tubes filling to approximately 1/2 volume and invert several times to mix.

3. Place tubes on a 10-20° slant for 45 min. for females and 60 min. for males. The blood should reach the top of the tube when slanted, and there should be an air-blood interface at the bottom of the tube.



4. After allowing to sit, stand the tubes upright (very gently) and wait about 5 min.

5. Remove plasma from sample with sterile pasteur pipette.
   A. Do cell count on hemacytometer by diluting in WBC pipette with 2% Acetic Acid sol'n. (Count the 4 large WBC squares and multiply the number by 5.0×10^4 to get number of cells/ml.)
   B. Do a differential on same hemacytometer prep. counting 100 WBCs.

6. Spin the RBC's that are remaining at 2000 rpm to recover as much plasma as possible.

7. According to counts, dilute the leukocyte suspension to 1×10^6 lymphocytes/ml. Use patients own serum obtained from No.6 as the diluent. (Can use $.5 \times 10^6$ lymphocytes/ml. and not change the results significantly)

Culture Technique

1. In a sterile 15 X 125mm culture tube add 2cc of MEM Media-Spinner Modified to which Pen-Strep (1cc/100 ml. MEM) and Glutamine (1cc/500 ml. MEM) have been added.

2. Add 1cc. of lymphocyte suspension to each culture tube.

3. Add antigens to culture tubes last. Keep one control tube.
   Antigens: A. Streptolysin O: 0.1 ml./culture

   B. Pokweed Mitogen: 0.05 ml./culture

   C. KLH: 0.1 ml./culture
   (Keyhole Limpet Hemocynanin)
   Three concentrations made and used: 3 mg/ml, 0.3 mg/ml, and 0.03 mg/ml (0.3 mg/ml gives best results)

   D. Veridase: 0.1 ml/culture
   Make up so have 0.6 - 1.3 mg/.1 ml.
   (Make up according to Streptocynase which is 20,000 units. Add 2cc NaCl making it 2,000 units. Dialize for 2 days against saline to remove preservatives. Then millipore and store in sterile containers. Order a large amount with the same lot number and store. Run a protein determination also.)

   E. Con A:

   F. Paytohemagglutinin M-Bacto: 0.05 ml/culture
   Add this antigen last.

4. Mix tubes on vortex mixer (slowly).

5. Loosen caps and put into humidified incubator that has 5% CO_2 (Hersh gauge set at 0.2) and 10% Air (Hersh gauge set at 4 and H_2O in line taken out with Pram-Oil Filter). CO_2 tank was 100% CO_2 - Liquified USP grade.

6. Incubate PHA cultures for 3 days; all others go for 5 days for DNA analysis.

^{3}H-Thymidine Uptake Procedure

1. Isolate lymphocytes.
2. To 10^{6} lymphocytes add 1 μc in 0.1cc of ^{3}H-Thymidine to each sample (culture). ^{3}H-Thymidine has been diluted with sterile water to 1.9 curies/millimole.
3. Incubate for 2 to 3 hours at 37°C.
4. Place in ice bath to chill 10-15 mins. Maintain everything cold from this point on.
5. Centrifuge (refrigerated centrifuge) at 1500-2000 for 10 mins. Discard supern.
6. Add 4cc cold saline, vortex, and centrifuge as above. Discard supern. (Can freeze samples here to save)
7. Add 4cc 5% TCA after thawing. Place on ice for 15 mins. and centrifuge. Discard supernatent.
8. Add 4cc 5% TCA again, vortex, and place on ice for 10 mins. Centrifuge and discard supern. (The button will be pinkish brown.)
9. Add 4cc methanol - to decolorize RBCs - vortex, and centrifuge. Discard supernatent. (May have to repeat for more decolorizing to reduce quenching.)
10. To the button add 0.5cc Soluene and vortex to resuspend.
11. Place in 60° water bath for 30 mins. (Can refrigerate now and store overnight)
12. Add 10 ml of scintillation fluid. (To transfer the ppt from the testtube to the counting vial, add only 5 ml of scin. fluid, transfer this to the counting vial, and then add the other 5 ml to the testtube to wash out any remaining ppt and add this to the counting vial.)
13. Cover samples for 30 mins. in the dark before counting.
14. Count for 50 mins. (Run blank of 0.5 ml soluene and scin. fluid.)

Reagent Supply Sources: For lymphocyte culturing technique

1. Dextran, (grade HH, Pyrogen free, 2 lb. Ave. M.W. 240,000)
   Pharmachem Corp

2. Penicillin-Streptomycin Sol'n (10,000 units Penicillin plus 10,000 mcg Streptomycin/ml) Unit Size: 20 ml. at 2.80 each bottle
   Cat. No. 514
   Gibco

3. Pokeweed Mitogen Unit size: 5ml. at 5.50 each bottle
   (Shipped on ice)
   Cat. No. 536
   Gibco

4. L-Glutamine (200mM)(100X), 29.2 mg/ml, Unit Size: 20 ml. at 3.50 each bottle
   Cat. No. 503
   (Shipped on ice)
   Gibco

5. S-MEM Minimum Essential Media (Eagle) (1X)
   with Earl's salts (Spinner modified)
   without L-Glutamine
   Unit size: 100 ml. at 2.30 each bottle Cat. No. 138
   Gibco

6. Bacto Streptolysin O Reagent
   6 bottles of 10 ml each for 7.50
   Cat. No. 0482-60-4
   Difco

7. Phytohemagglutinin M-Bacto,
   6 bottles of 5 ml each for 21.25
   Cat. No. 0528-57-5
   Difco

8. KLH (Keyhole Limpet Hemocynanin)
   Even immunosuppressed can be stimulated with this. Divers may become immune to this. Associated form used MW - 180,000
   Three concentrations made and used(See Procedure)
   Procedure for making up in Immunology by Campbell (uses live limpets)
   Pacific Bio. Marine Supply
   Box 536 Denice, Calif. Order FOB Los Angeles
   (They may have prepared KLH also)

9. Veridase (for intervenous injection)
   Make up according to Streptocynase which is 20,000 units
   Add: 2cc NaCl making it 2,000 units. Dialize for 2 days against saline to remove preservatives. Then millipore and store in sterile containers. Order a bunch with same lot no. and store. Run a protein determination also. Use .1ml/culture of .6-1.3 mg/.1 ml

Scintillation fluid preparation:

Packard Products used.

PPO	5.0 grams
POPOP	0.1 gram

Add tolune slowly to 1 liter.

LYMPHOCYTE SEPARATION

Ficol Method
(Dr. Hersh's Lab)

Coat all glassware with Siliclad to prevent lymphocytes from sticking.

Preparation of Reagents:

1. Ficoll: 100 grams to 1100 cc. d.H_2O (Makes 9% sol'n)
   Autoclave 15 mins. on liquid cycle to dissolve and sterilize. Keep refrigerated.
2. Hypaic: Dissolve 50 mls. in 83 mls. sterile H_2O, rinsing bottle thoroughly. (Makes a 33.29% sol'n.)
   Keep refrigerated.
3. Tris-Buffer (for removing RBC's from prep.):
   a. 4.1188 grams Tris Trizma base dissolved in 200 ml. d.H_2O
   b. pH to 7.65 with HCl
   c. 14.94 grams NH_4Cl dissolved in 1800 ml. d.H_2O
   d. Add Tris
   e. pH to 7.2 with HCl
   f. before using warm it to 37°C.

PROCEDURE:

1. Defibrinate blood and dilute 3:1 with sterile saline
2. Layer blood over ficol (approx. 3 part blood to 1 parts ficol, or as to give blood a good distance to travel down to bottom of tube)
3. Centrifuge at 500 g or 1500 rpm (check chart for Fisher centrifuge) for 40 min. or 2500 for 15 min. (gives more RBC contamination on higher speed and shorter time)
4. With pipette, combine Lymphocyte layers (filling tubes 1/2 full) Usually get 90% lymphocytes and 10% monocytes and polymorphs
5. Fill tube containing lymphocytes with Tris, mix, and let sit for 10 mins. (If need to remove RBC's) Spin at 1000 for 10 mins. to get pellet. Tris again if RBC's are still present
6. Rinse cells about 2X with media to remove Tris and Ficol.

Ficol Separation (con'd.)

7. If do not Tris, dilute the lymphocytes 1 part cell suspension to 1 part MEM so that when spun to get button cells will all spin down and the gradient will no longer be present.
8. Add 1 ml. cells (1×10^6) to 2 mls. MEM
After culturing should have no less than 96% viability.

Ficol - Hypaic Solution : (Make fresh each day)

1 cc. Hypaic to 2.4 cc. Ficol

HW-28-J

HYPAQUE®-M, 90%

Brand of

SODIUM AND MEGLUMINE DIATRIZOATES

Sterile Aqueous Injection 90% (weight/volume)

A Radiopaque Medium for Angiocardiography, Aortography, Angiography, Urography, and Hysterosalpingography

DESCRIPTION

Hypaque-M, 90 per cent is a sterile aqueous solution containing 30 per cent (weight/volume) of the sodium salt and 60 per cent (weight/volume) of the meglumine salt of 3,5-diacetamido-2,4,6-triiodobenzoic acid. Each ml. contains approximately 462 mg. of organically-bound iodine and 0.47 meq. (or 10.9 mg.) sodium per ml. The solution is hypertonic and has a viscosity of approximately 18.7 cps at 37°C. The pH is adjusted with NaOH or HCl. Calcium disodium edetate 1:10,000 has been added as a sequestering stabilizing agent. The solution may be autoclaved. It should be protected from strong light. At body temperature the solution is clear and colorless to pale straw color. Crystals may form in the solution on cooling; they are readily redissolved on warming; the solution, however, should be administered at body temperature.

INDICATIONS, General

A radiopaque medium for angiocardiography, aortography, angiography, urography, and hysterosalpingography.

CONTRAINDICATIONS, General

Do not use Hypaque-M, 90 per cent for myelography. Injection of even a small amount into the subarachnoid space may produce convulsions and result in fatality.

Hypaque-M, 90 per cent should not be injected directly into the carotid or vertebral arteries.

WARNINGS, General

Do not use Hypaque sodium for myelography. Injection of even a small amount into the subarachnoid space may produce convulsions and result in fatality. Epidural placement is not advised.

Intravascular administration of contrast media may promote sickling in patients who are homozygous for sickle cell disease.

Administration of radiopaque materials in patients known or suspected to have pheochromocytoma should be performed with extreme caution. If, in the opinion of the physician, the possible benefits of such procedures outweigh the considered risks, the amount of radiopaque medium injected should be kept to an absolute minimum. The

Suggested Hersh Values:

1. 0 Hour in Vivo stimulation - CPM
   300 - 1,000 for nonreactive or unstimulated
   Above 1,000 for reactive

2. PHA ranges - THA-72 hrs. - CPM

   A. Stimulated: Range: 15,000 - 100,000 (15,000 considered extreme low)
   Ave: 30,000 - 40,000

   B. Unstimulated Controls: Range: 400 - 600

To Convert CPM to DPM:

$$DPM = \frac{CPM}{\text{efficiency (using stds.)}}$$

Kathy's calculations correcting CPM's to 1 X 10^6 viable cells is:

$$DPM/1 \times 10^6 \text{ viable cells} = \frac{\frac{\text{Total Count}}{\text{Time}} - \text{Background CPM} \times 100}{\text{No. of cells counted} \times 10^6 \times \% \text{ Viability}}$$

$$DPM/1 \times 10^6 \text{ viable cells} = \frac{CPM/1 \times 10^6}{\% \text{ Efficiency}}$$

AMMONIUM CHLORIDE RECIPE FOR REMOVING RBC'S

1. Prepare Tris Buffer:

   20.594 grams of Tris Base/liter d.H_2O. Adjust to pH 7.65 with 1N HCL.

2. Prepare Ammonium Chloride Sol'n: 0.83 %

   0.83 grams ammonium chloride/100 cc. d.H_2O.

3. Prepare a mixture of the above two solutions:

   A. Add 1 volume of Tris Buffer to 9 volumes of .83% aqueous NH_4Cl.

   B. Adjust mixture to pH 7.2 with 1 N HCL.

   C. For Sterile Work: Millipore filter with .22 filters. Store at room temp. in small quantities.

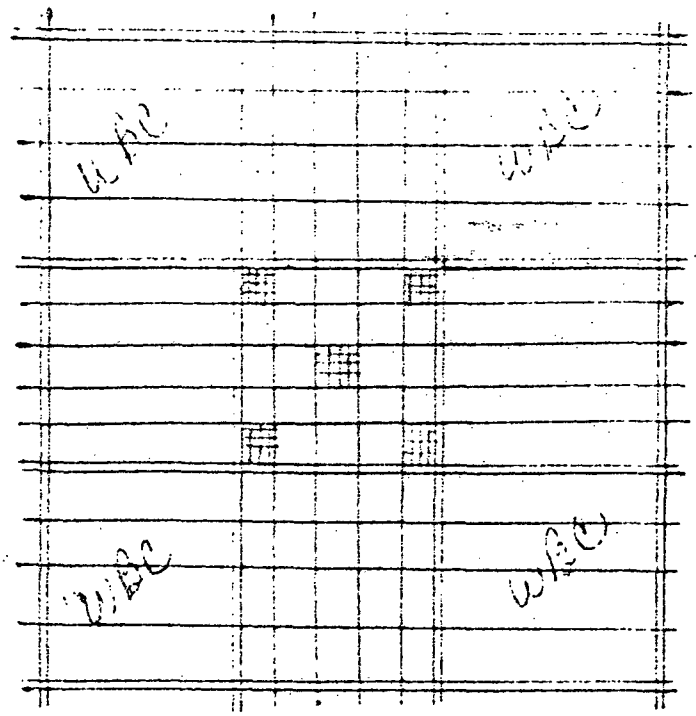
To Use:

1. If not too many RBC's in pellet, add to top of tube (NH_4Cl)
2. Resuspend
3. Let sit 5 mins.
4. Spin at 1,000 per min. to settle lymphocytes and leave debris behind.

Reference: Boyle, W., Transplantation, vol. 6, p. 761, 1968.

Counting # WBC's using diluting pipette:

$$\frac{\#\text{ cells counted in 4 WBC squares}}{} \times 50 \times 10^3 = \#\text{ WBC's/ml. of sample}$$



Formula for counting cell cultures (without using WBC diluting pipette):

ives total # of cells present in sample

$$\frac{\#\text{cells counted} \times \text{volume cells are in}}{.1 \times \#\text{large squares counted}} \times 10^3 = \#\text{cells in culture}$$

Trypan Blue Stain: (0.4 %) in normal saline or order from GIBCO #525
$1.75/100 cc. ($10.00 - minimum order)

Tris - Buffer: Trizma Base Reagent grade No. T-1503
1 kilogram molecular equiv. wt. 121.1
Sigma Chem. Co.

Reagents Used: Check list

1. Sterile isotonic saline
2. Dextran, (grade 4H, Pyrogen free, 2 lb. Ave. M.W. 240,000)
3. 2% HAc for cell counts
4. MEM Media - Spinner modified
5. Pen-Strep
6. Glutamine
7. Sterile distilled H_2O (small bottles)
8. 5% TCA
9. Methanol
10. Soluene
11. Toluene (Scint. grade)
12. ^{3}H-Thymidine (1.9 curies/millimole)
13. Streptolysin O
14. PHA-M
15. PWM
16. Con A
17. Veridase
18. KLH (Keyhole Limpet Hemocyanin)

MEM Eagle, Earle's Base, Dehydrated, is a nonsterile powder containing all of the ingredients of the MEM Eagle, Earle's base formula including glutamine, but without sodium bicarbonate.

Prepare a working solution by adding the powder from one or more containers to an appropriate amount of deionized water. Stir at room temperature until thoroughly dissolved (15 to 20 minutes). Sterilize by filtration through an appropriate bacteriological filter. Finally, add 29.4 ml. of 7.5% Sodium Bicarbonate Solution per liter of medium.

MINIMUM ESSENTIAL MEDIUM EAGLE, SPINNER MODIFIED

100 ml. No. 51054

500 ml. No. 51058

Ingredients Per Liter

l-Arginine HCl	126.98	mg
l-Cystine	24	mg
l-Glutamine	292	mg
l-Histidine HCl • H_2O	41.88	mg
l-Leucine	52	mg
l-Isoleucine	52	mg
l-Lysine HCl	58	mg
l-Methionine	15	mg
l-Phenylalanine	32	mg
l-Threonine	48	mg
l-Tryptophan	10	mg
l-Tyrosine	36	mg
l-Valine	46	mg
Choline Chloride	1	mg
Biotin	1	mg
Folic Acid	1	mg
Inositol	2	mg
Ca-D-Pantothenate	1	mg
Pyridoxal HCl	1	mg
Thiamine HCl	1	mg
Nicotinamide	1	mg
Riboflavin	0.1	mg
Sodium Chloride	6.8	g
Potassium Chloride	0.4	g
Magnesium Sulfate	0.118	g
Dextrose	1	g
Monosodium Phosphate • H_2O	1.33	g
Phenol Red	0.005	g
Sodium Bicarbonate	2	g

LYMPHOCYTE SEPARATION BY HAND METHOD

1. Type the blood sample. Pour it into a graduated centrifube tube and add 2 ml. 5 %PVP/10 ml. blood.
2. Mix by inverting tube 5X.
3. Centrifuge on setting 3/4 on ICC centrifuge for 10 mins.
4. Save 1 fisher tube of serum for later.
5. Remove buffy coat and put in fisher tube.
6. Centrifuge at 2500g for 2 mins.
7. Remove buffy coat and place into cup (take some of the serum with it) and add antisera in equal volume.
8. Place on shaker under a light source for 5 mins.
9. Let stand for 5 mins.
10. Place liquid from cup in a fisher tube, leaving afflutinated RBC's behind.
11. Do a "quickie" spin on fisher tube to remove any RBC's.
12. Place supernatant in a clean tube and spin at 250^g for 2 mins.
13. Resuspend the pellet in a 1:1 solution of patient's serum and Hanks.
14. Place on straw column.
15. Incubate at 37°C in waterbath for 10 mins.
16. Flush straw with McCoy's medium into approx. 2-4 fisher tubes.
17. Spin tubes at 250^g for 2 mins.
18. Resuspend pellets (combining them in one tube) in 1 ml. McCoy's.
19. Count on Hemacytometer. Use 5 small squares.

Preparing Phosphate Buffered Saline Solution (PBS): pH 7.4

Stock A:	$NaH_2PO_4 \cdot H_2O$	46gm/1Liter 92gm/2 Liters
Stock B:	Na_2HPO_4	47.3gm/1 Liter 94.6gm/2 Liters

For 6 Liters PBS:

NaCl	52.26 gms.
Stock (A)	9.6 cc.
Stock (B)	50.4 cc.

To 6 Liters with $d.H_2O$

For 8 Liters PBS:

NaCl	69.68 gms.
Stock (A)	12.8 cc.
Stock (B)	67.2 cc.

ACRIDINE ORANGE STAINING PROCEDURE FOR LYMPHOCYTE CYTOFLUROGRAPH STUDIES

1. Separate lymphocytes by standard procedure using Technicon Separator and count.

2. Centrifuge, withdraw supernatent and resuspend cells in 2 ml. buffered formalin (cold) *

3. Refrigerate for 3.5 hours (note the exact time)

4. Add cells (still suspended in formalin) into Cold Acridine Orange at 0.5×10^6 cells/2 ml. stain. Store in refrigerator in a dark stoppered vessel. Record time cells added to stain.

* The amount of formalin needed is arbitrary depending on the number of cells stained.

FLUORESCENT ANTIBODY STAINING OF LYMPHOCYTES

Calculating Results

Cytoflurograph: On 2-8 window readings - add M, G, and A and see if they equal T. Calculate what % of T it is.

Fl. Microscope: Calculate % fluorescence (from scope readings by putting the number of fluorescing lymphocytes over the total number of lymphocytes counted) on T, M, G, and A samples. Add M, G, and A to see if they equal T. Calculate what % of T it is.

Viability: Get a % viability and % lymphocytes in each prep. from the hemacytometer readings.

Take the cytoflurograph readings (%) for T, G, M, and A, and using the % of lymphocytes in the differential, calculate the % fluorescence on the cytoflurograph.

$$\frac{\text{Cytoflurograph}}{\text{Lymphocytes}} = \%$$ fluorescence

Add M, G, and A to see if equal T. Compare to % found from microscope.

FLUORESCENT ANTIBODY STAINING OF LYMPHOCYTES

for Fl. Microscopy and cytoflurograph

*

1. Spin down samples in Fisher tubes at 2500 rpm for 2 mins. (or 2000 g for 3 mins.)

2. Wash each sample with cold PBS X 3.

3. On third wash divide each sample into 5 parts (.2 ml each: marked unstained, Total anti Ig, anti IgM, anti IgG, and anti IgA)

4. Spin samples down and withdraw supernatant

5. In each subsample add 2-3 drops of appropriate stain to pellet and mix thoroughly, but gently. Do not make bubbles. Use 1:8 dilution of stains.

6. Place samples in refrigerator for 30 mins.

7. Top each tube with cold PBS and mix. (This is wash 1)

8. Spin cells at 2500 rpm for 2 mins. Wash X 2 with cold PBS

9. Then add 1 ml. PBS to samples and mix.

10. Prepare 1 tube with 9 mls. cold PBS and 4 tubes with 4 mls. cold PBS each to use in next step. Label as in step 3.

11. When placing 1 ml lymphocyte stained samples in tubes prepared in step 10, be sure to leave a few drops in bottom of fisher tubes to use later.

    Place 1 ml. of unstained into 9 mls. cold PBS

    Place 1 ml. of Total into 4 mls. cold PBS

    Place 1 ml. of IgM into 4 mls. cold PBS

    Place 1 ml. of IgG into 4 mls. cold PBS

    Place 1 ml. of IgA into 4 mls. cold PBS

    Mix well. Samples are now ready to be read on cytoflurograph.

12. Using the remainder of lymphocyte sample in bottom of fisher tubes, take a drop and place it on a glass slide (slides used for fl. microscopy) and coverslip with a 1 1/2 coverslip. Seal with clear nailpolish. Store slides in a dark, cool place (refrig.) until they are read.

    Take another drop and adding trypan blue, mix together and place on a hemacytometer. Do a viability reading and also a differential.

* Use lymphocytes that have been separated with Technicon Separator, preferably fresh, but can be used up to 24 hrs. old.

LYMPHOCYTE CULTURING TECHNIQUE

All sterile supplies must be used.

1. Label culture tubes as to the sample number and the antigen to be used.
2. Dilute the lymphocyte sample to 1.0×10^6 cells/ml. with person's own serum or Fetal calf serum which has been inactivated previously at $56^{\circ}C$ for 30 mins.
3. Into each culture tube place 2 ml. of MEM to which pen-strep and glutamine have been added.
4. Mix the lymphocyte sample well and add 1 ml. of the sample to the culture tubes, making a total of 3 mls. (33 % is serum).
5. Add antigens.
6. Mix well and loosen caps.
7. Place in an incubator at $37^{\circ}C$ (see Dextran separation technique). Allow cultures to go 3 days for PHA and 5 days for Pokeweed Mitogen. (They may go a few days longer if necessary, for convenience)
8. Harvest (can now use non-sterile supplies) by removing the supernatant (the cells will be at bottom of tube) and add 3 ml. fresh MEM.
9. Do a viability count with trypan blue if necessary.
10. Lymphocytes are now ready for ^3H-thymidine procedure or whatever other test is to be preformed.

LYMPHOCYTE SEPARATION PROCEDURE (For the Mouse)

1. Collect 4-5 mls. total heparinized blood by heart punctures (will need 7-10 mice).
2. Centrifuge blood in an International Clinical Centrifuge at 3/4 full speed for 10 mins. (a little longer if necessary to make a good buffy coat layer)
3. Generously transfer buffy coat to a Fisher Centrifuge Tube, mix, and centrifuge at 2500g. for 2 mins. in a Fisher Model 59 centrifuge.
4. Transfer buffy coat (with minimal red cells) to a 5 ml. cup containing an equal vol. of anti-mouse serum which has been diluted 1:2 (in PBS). Mix well.
5. Place cup on shaker under a light for 5 mins. (may take longer) and then allow to stand another 5 mins. or so.
6. Remove agglutinated red cells by low speed centrifugation (Fisher model 59) at 1000g. for 5 secs.
7. Centrifuge supernatant at 2500g. for 2 mins. (Will settle all cells but the platelets.)
8. Resuspend cells in 1 ml. of a 1:1 mixture of mouse serum (saved from sample of blood) and Hanks BSS.
9. Optional: Quick spin (1000g. for 5 secs.) to remove remaining RBC's, if present.
10. Place suspension on a 4-inch column of coated glass beads.
11. Stopper the straw at the lower end and incubate in a 37°C water bath for 10 mins.
12. Flush lymphocytes into several Fisher tubes with McCoy's 5a Medium, modified with 30% fetal calf serum.
13. Centrifuge tubes at 2500g. for 2 mins. to settle lymphocytes.
14. Remove supernatants and combine all lymphocytes into 1 ml. of McCoys.
15. Mix well and view under a hemacytometer.
16. Adjust count with McCoys to 1.0×10^6 lymphocytes/ml.

NEEDED FOR LYMPHOCYTE SEPARATION AND ^{3}H-THYMIDINE UPTAKE PROCEDURES:

Column: Coat glass beads by covering them with a 1% sol'n. of gum arabic and allowing them to dry thoroughly in a glass drying oven. Break beads apart by grinding in a mortar and pestle dish. Plug drinking straw with glass wool and fill straw to approx. 3" from top with the coated glass beads.

Working Stock of ^{3}H-thymidine:

Original stock has 2 μci/100 (in 0.5 ml.)
Add 12.5 ml. sterile H_2O to the original stock, rinsing stock vial with the water thoroughly into a clean larger vial. One squirt with a 100λ pipette will give 2 microcuries.

Rabbit Anti-mouse Serum:

Prepared by inoculating rabbit with pure mouse RBC's and then using the rabbit serum (after it has been inactivated at 56°C for 30 mins.) to agglutinate mouse RBC's.

Formulas for Calculating* of cells/ml. using hemacytometer:

(A) If Counting one large WBC square use:

$$\frac{\text{ct. X vol.}}{.1 \text{ X large squares counted}} \quad \text{X} \quad \left(\begin{array}{c}10^3 \\ \text{converts} \\ \text{mm to ml}\end{array}\right) = \text{cts./ml.}$$

*(B) If counting RBC area, e.g. 5 small squares in large center square use:

$$\frac{\text{ct.}}{.2 \left(\begin{array}{l}\text{is .5 of} \\ \text{large square}\end{array}\right)} \quad \text{X vol. X 10 X } 10^3 = \text{cts./ml.}$$

(C) Above (B) written differently:

$$\frac{\text{ct. X vol.}}{.1 \text{ X } .2} \quad \text{X } 10^3 = \text{cts/ml.}$$

Solutions

PVP (5%)

25 grams polyvinylpyrolidinone K-90
1 sterile 500 cc. bottle saline

Add PVP and roll gently; let stand overnight and mix gently again.

Lechtin

1/2 cup seeds
Cover seeds with pH 7.2 PBS in blender. Blend until mashed.
Put in beaker 1 to 2 days. (1 hour 10 min.)
Centrifuge ale precipitate
Test against an O cell
Store in freezer at -20°C.

Heparin

One bottle Na Heparin
Add 100 ml. sterile saline
Store in refrigerator
Use .4 ccs/20 ccs. blood

EDURE FOR COLLECTION AND PROCESSING OF MOUSE EXUDATES

Prep mice by I.P. injection of 1.0 cc of 0.1 o/o glycogen in sterile saline. (Keep refrigerated; discard when contaminated.)

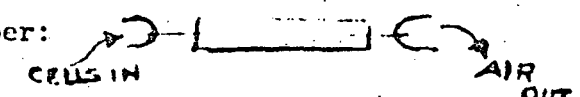
Allow cells to immigrate into the peritoneal cavity for 4 hrs. Anesthetize the mice with Metofane and inject 5.0 cc sterile saline I.P. with a 20G needle. Remove the syringe, leaving the needle in place. Collect the exudate into clean, dry, siliconized conical cent tubes.

Pellet the cells by centrifugation @ 1500 rpm for 10 minutes. Discard supernatant.

Resuspend cells in tissue culture medium by gentle vortexing.

After cells are completely resuspended, remove aliquots for total WBC, diff, and viability.

Introduce cells suspended in medium into chamber:



Remove needles and place chamber in 37° water bath (held above water level on platform) for 15 minutes.

Remove chambers at 15 minute intervals and observe cells using phase microscopy.

Record damaged cells, vacuolated, rounded up, floating or obviously viable cells that are well flattened, extended and showing evidence of movement.

Initially, we will be concerned with cell collection, preparation, and preservation. The effects of such variables as time elapsed after preparation (before collection), type of media, etc. will be evaluated.

Checking Fluorescent Antisera for Fluorescent Specificity (Fluorescent A.B. staining with Goat Antisera)

Remove liver and spleen from mouse: preferably mouse that has had some disease (will have more antibodies)

Liver - used as control tissue

Spleen - where immunoglobulins in mouse are produced

1. Take fluorescent antisera and thaw. Remove with syringe and place in Fisher tubes. Label tubes.
2. Spin tubes at 5000g for 5 mins. to settle any ppt. present in antisera.
3. Remove serum ppt. and put serum in clean vials and relabel vials. Check to see if vials are clean first.
4. Re-refrigerate vials and refreeze any you won't be using immediately.

Use Direct Staining Technique: Goat antisera undiluted gave highest (4+ fluorescence)

FLUORESCENT STAINING

Direct Technique:

Mouse tissue: add fl. antisera - goat antisera against mouse gamma globulin

Human cells: add fl. antisera to:

Human immune globulin G (IgG)
Human immune globulin A (IgA)
Human immune globulin M (IgM)

> all of these together would be human gamma globulin (whole)

(These bought commercially in pure form. Can test them for purity with Immunoelectrophoresis.)

Indirect Technique:
(sandwich technique)

First apply antisera (unlabelled)

Example:

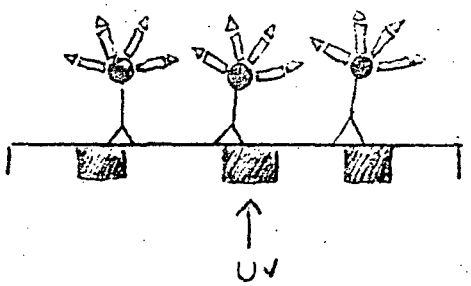
Take mouse blood smear (mouse has a disease- malaria)

Add antisera (sera from a diseased mouse) and incubate 30 mins.

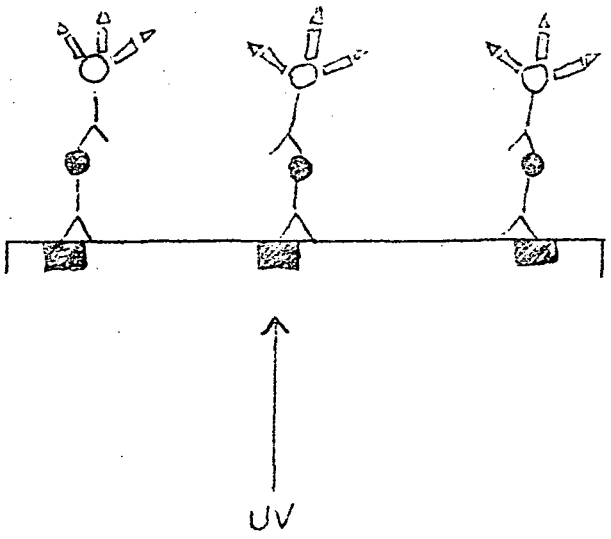
Then add Fluorescent antisera against mouse gamma globulin and incubate 30 mins.

Immunoflourescence: Diagrahms

Direct Test: Detects antigen



Indirect Test: Detects antigen and/or Immunoglobulin (antibody) after its reaction with antigen



Preparation of Slides for Immuno Fluorescence Studies

Direct Staining

1. 30 secs. in 95% ETOH - wash off with PBS in wash bottle (or pour from bottle)
2. 5 mins. in PBS - 2X
3. Wash off slide with PBS
4. dry - except the tissue
5. Supply FICA anti-sera
6. Incubate 30 mins. in moisture chamber
7. Repeat steps 2,3,4,5
8. Supply cover slips with buffered glycerine

Use cold PBS

Buffered glycerine - 20cc. glycerine
1 drop PBS

Indirect Staining Technique

1. Mark off area of tissue to be stained with marker (ink pen)
2. Dip in 95% ETOH for 30 secs.
3. 5 mins. in PBS - 2X after rinsing slide with wash bottle of PBS
4. Wipe off slide around marked area
5. Add a drop of aniesera
6. Incubate 30 mins. in moisture chamber
7. 5 mins. in PBS - 2X
8. Wipe around marked area
9. Add one drop of fl. antisera
10. 30 mins. in moisture chamber - then rinse with rinse with squirt bottle
11. PBS wash - 2X
12. Rinse off with squirt bottle
13. Wipe around marked area
14. Coverslip with buffered glycerine

Fluorescent Antisera dilutions: Make dilutions with PBS, pH 7.4

Fluorescent antisera to mouse gamma globulin (made in goats)
Use diluted 1:4

Human Fl. antisera
Use diluted 1:2

ABSORBING FLUORESCENT ANTISERA

1 cc. Sera

↓

Add 10 mg. mouse liver powder

↓

Sit for 30 mins. at room temp.

↓

Stir every 10 mins. with stick

↓

Spin as hard as possible (5 mins.) X 3

1. Use Reabsorbed antisera on tissue slides
   1:4 dil., 1:2 dil.
2. Examine slides for optimum staining dilution

ACRIDINE ORANGE STAINING TECHNIQUE

Fixation of slides:

ETOH-ether: ethyl alcohol(abs.)
1:1 for 15 mins.

dilute with NaCl

- 80% alcohol: 5 mins. (33.7 cc. ⟶ 40 cc.)
- 70% alcohol: 5 mins. (29.5 cc. ⟶ 40 cc.)
- 50% alcohol: 5 mins. (21.1 cc. ⟶ 40 cc.)

Can store slides in NaCl before staining

Staining:

Acridine Orange: Dilute 1:10 with McIlvaine buffer mixture, pH 3.8 or Phosphate Buffered Saline (PBS) pH 4.6.
Filter mixture before using.

1. Immerse slides in diluted AO for 5 secs. with agitation.
2. Immerse slides for 12 secs. in 2% ethanol in isotonic saline (2 ml. ethanol + 93 ml. saline)
3. Rinse for 5 secs. in isotonic saline and coverslip wet. With dispopipette put drop of NaCl at edge of coverslip to keep it wet. Store in petri dish.

Timing variations that give good results: Using PBS

5" in AO and 20" in 2% ETOH in NaCl

5" in AO and 12" in 2% ETOH in NaCl

3" in AO and 12" in 2% ETOH in NaCl

Acridine Orange Staining

Phosphate Buffered Saline

Buffers:

Stock A:	$NaH_2PO_4 \cdot H_2O$	27.6 grams (to 1 liter)
Stock B:	$Na_2HPO_4 \cdot 2H_2O$	35.61 grams (to 1 liter)

PBS: for 1 liter (distilled H_2O)

8.77 gms. NaCl
19.5 ml. Stock A
30.5 ml. Stock B

(See Reprints)

Buffer Solution McLlvaine, pH 3.8

Sodium Phosphate	10.08 grams
Citric Acid	13.56 grams
Water, distilled..QS..	1.0 Liter
Dowicide, 1%	5.0 ml.

STAINING PROCEDURE FOR CYTOSCAN

1. Fix in 95% Ethanol - 15 mins.
2. Wash in H_2O
3. Dry
4. 100% Ethanol
5. 95%
6. 95%
7. 80%
8. 70%
9. H & E Stain - 1 min.
10. Acid OH - Dip rapidly
11. H_2O
12. H_2O
13. Ammonia
14. H_2O
15. H_2O
16. 70% ETOH
17. Eosin - 25 secs.
18. 70% OH
19. 80%
20. 95%
21. 100%
22. Dry slides
23. Mount in Glycerin

Wright's Stain:

1. Stain same day smears are made
2. Cover smear with Wright's Stain - let stand 2 mins. (do not allow to ppt. through evap.)
3. Add equal amt. buffer sol'n. (pH 6.4)
   Distribute over whole slide - none should run off edges
4. Stain and buffer mixed by blowing on surface (mixture should show a brassy sheen)
5. Let stand 5 mins.
6. Wash stain - run stream H_2O directly into center of slide for 30 secs.
7. Wipe off back of slide with cloth
8. Stand on end and let dry in air
9. If stain ppts. cover slide with 95 % ETOH and wash immediately
10. If stain is not deep enoughh, restain

Gram Stain:

1. With pasteur pipet put a little of culture on slide
2. Allow to air dry
3. Heat fix over flame (just until slide becomes hot)
4. Place slide on rach and flood with crystal violet
   Leave on 1 min. Then shake off
5. Wash off completely with squirt bottle of H_2O
6. Add 95 % ETOH
   Leave on approx. 2-5 secs. (until blue stops coming out of the prep.)
   Wash off immediately with H_2O
7. Add safranin - leave on 30 secs.
   Wash off and blot dry.

Preparation of Immunoelectrophoresis Plates

1. Dilute LKB buffer 1:4 (75:225) with d.H_2O (300 ml. total)
2. Need 1 1/2% Noble Agar Sol'n
   Prepare by adding 4 1/2 gms. agar to the above 300 cc. buffer.
3. 10 mgms. of methiolate/100cc.
4. Put in boiling H_2O (make a double boiler) until it goes into sol'n. (45 mins.). Shake inbetween. Cool for 1/2 hr. at 56°C.
5. Number the slides and put in plastic holder.
6. Put 12 cc. on each side of slide rack. Fill edges first and then slides.
7. Let set until hard.
8. Put in metal holder and put in moisture chamber and refrigerate until used.

LKB buffer: 65 gm. Na Acetate and 103 gm. Na Barbitol
18.4 gm. Barbitol (add to water) → in 10 liters (Keep refrigerated)

Slides: Gelman slides 51459 or any without frosted end will do.

Chamber: 700 ml. LKB put in each side of chamber. Wicks kept in refrigerator. Drape them on top of ends of agar slides and into troughs. With dispopipette wet wicks until very wet.

Procedure for Immunoelectrophoresis:

1. Cut holes and wells with punch set. Then suction agar out of holes.
2. Apply sample(s) to holes.
3. Place agar slides in chamber and apply wicks to each end.
4. Run for 45-60 mins. at 250 volts pulling 12 MA.
5. Turn off maching and remove slides. Suction agar from troughs.
6. Add antisera to wells (fill them) and let slides stand at room temp. in moisture chamber overnight.

Macrophage Study Procedure:

1. Do four implants per study: one extra in case one mouse dies.

   Day 0: day of implant
   Day 1: day 1 after implant
   Day 2: day 2 after implant
   Day 3: day 3 after implant

   Each day an implant is removed and its contents studied

2. .1 ml. parasitised blood (P.b.) is injected into chamber with a 26g. needle.
   First rinse blood 2X in PBS and dilute u/diluting (RBC) pipette ~~to a pink solution~~. Then count number of RBC's per .1 ml. Also determine parasitemia from tail smears of mice parasitised blood was taken from. Figure the number of parasited cells that will be injected.
   Add a known # of macrophages to the diluent.
3. After the .1 ml. blood is injected into the chamber seal chamber hole with glue. Place implant into peritoneal cavity of mouse using semi-sterile technique.

   Things that can happen to alter results:

   1. Volume of liquid change: % parasitemia won't change but number of RBC's will in a smaller volume.
   2. Lysis of some of the cells.
   3. Avoid placing chamber on a damp flat surface after removing from the mouse as some of the fluid may be lost. Be sure to place in a moisture chamber on sticks away from the moist tissue paper.
   4. When placing into peritoneal cavity of the mouse avoid contacting chamber with alcohol or other antiseptic used to wipe mouse as cells might be killed if it is absorbed into chamber.
   5. Do not rupture sides of chamber when inserting and removing from mouse.

4. After removing chamber from mouse cavity examine by withdrawing fluid with 26g. needle and 1cc. syringe and checking volume recovered. The vol. recovered from 0.1cc. is around 0.06cc. Count number of cells on hemacytometer.
   (Fribrin usually forms in the chambers and heparin can be injected into the mice when chambers are implanted to prevent this)
5. From the hemacytomer cell count figure how many RBC's were recovered out of the number injected. (Account for volume change)

Macrophage study procedure (con'd)

Heparin injections to be given:

Day 0: .1 cc. intraperitoneally
Day 1: .15 (morning), .05 (afternoon)
Day 2: .1 cc. (morning)

In the macrophage study 8 to 12 mice will be used. There will be a control group of mice (chambers will contain parasitised blood only) and a test group (chambers will contain parasitised blood and macrophages). Each mouse will have two chamber implants for a duplicate.

Preparing the macrophages:

The macrophages are obtained from the ascites fluid formed in mice injected with freund's adjuvent.

Procedure: Inject 10 mice with 1 cc. complete Freund's adjuvent. One week later inject mice again with incomplete Freund's adjuvent. An ascites will form which can be drained from the peritoneal cavity.

Mouse numbering system (mark with picric acid

Preparing and Testing Rabbit Anti-Mouse Serum

Injecting rabbits:

Obtain 1 1/2 - 2 cc. normal mouse blood and centrifuge for 10 mins. Remove serum and buffy coat and rinse red cells 4X in NaCl. Dilute to 4.0 mls. and count number of RBC's/ml. on hemacytometer. Inject two rabbits with 0.5 cc. in each shoulder and each hip. (Total of 4.0 ml. injected) Boost (repeat above procedure) for 3 to 4 weeks (once a week) until rabbits have had sufficient time to produce a high level of antibody against the red cells of the mouse.

Bleeding rabbits:

1. Remove rabbit from cage and place on table top. Wrap securely in a cloth.
2. Shave outer edge of rabbits ear near the base and apply vaseline. Shave tip of ear and apply xylene. This will cause irritation and the vein will engorge with blood.
3. With a sharp pointed razor blade nic the vein where the vaseline was applied and collect blood in tubes. About 30 cc. from an average size rabbit is good.
4. After finishing collection unwrap rabbit and rinse xylene from the ear. Press ear where cut for several minutes until bleeding stops.
5. Before returning rabbit to cage be sure to remark rabbits ear.

Testing Activity of Serum: agglutination ability of rabbit anti-mouse serum

1. Inactivate the rabbit serum by heating at 56°C for 30 mins.
2. Bleed out a normal mouse and rinse the RBC's in NaCl 2 X. Put cells in 2 ml. Nacl.
   Add .1 cc. cells to each of 7 small tubes:

1 2	no antisera	
3	.1 ml undil. antisera	
4	.1 ml antisera (diluted 1:1 with NaCl)	1:2 dil.
5	.1 ml of 1:2 (dil. 1:1 with NaCl)	1:4 dil.
6		1:8 dil.
7	.1 ml of 1:10 antisera (.1 conc. plus .9 PBS)	1:10 dil.

Look at agglutination immediately. Put in 37°C water bath for 10 mins. and read again. Grade from 0 to 4+.

UCSF
Culture Preparations

LYMPHOCYTE PREPARATION

A. Blood--0.2 ml of phenol free heparin per 10 ml whole blood

1. Divide blood to be used into sterile Fisher tubes and cap.

2. Centrifuge for two minutes at 3500 G in a Fisher centrifuge.

3. Remove the buffy coat and place in a Fisher tube containing anti AB or Anti H depending on blood type.

   a. Blood groups A, B or AB--use 0.25 ml Anti AB plus 0.05 ml Anti H.

   b. Blood group O--use 0.3 ml Anti H.

4. Agglutinate red blood cells by inverting tubes approximately 8 times per minute for two to five minutes.

5. Remove agglutinated red cells by spinning for three seconds at 1000 G in a Fisher centrifuge.

6. Transfer the supernatant to a sterile Beckman tube and spin full speed for 10 seconds in a Beckman centrifuge to agglutinate any remaining red cells.

7. Discard the supernatant and gently resuspend the cells in TC199 with 20% AB serum, with a Pasteur pipette, freeing the leukocytes from the clumped cells.

8. Remove the residual red cells with a one third speed, two second spin. Control speed by a transformer. Transfer the supernatant, containing leukocytes, and centrifuge at full speed for 10 seconds to wash out Anti AB serum. Resuspend the sedimented leukocytes in 0.1 ml of normal AB human serum and 0.1 ml of TC 199/20 % AB serum medium.

9. Place the solution on a three quarter-inch column of glass beads contained in a nylon-plugged plastic drinking straw. (The beads have been precoated with 1% gum arabic and dried.)

10. Incubate at 37°C for two to three minutes.

11. Flush out lymphocytes with 1.5 ml TC 199/AB serum medium into a Fisher tube and centrifuge at 1000 G for 2 minutes.

12. Remove supernatant and resuspend cells in approximately 1 ml of TC 199. Centrifuge at 1000 G for 2 minutes.

13. Repeat wash once more. After removing supernatant as completely as possible, add 1 ml TC 199/20% AB serum media to tube and resuspend cells.

14. Do a cell count using Unopette and hemacytometer to determine number of lymphocytes. Calculate % of polys present. Cells are then ready to be cultured

CULTURE PREPARATION

A. Pokeweed mitogen (PWM). Reconstitute contents of vial with 5 ml of sterile distilled water. Amount to be used is the equivalent of 0.1 ml per 10 ml of culture fluid. Adjust according to size of culture to be set up.

1. Using sterile Fisher tubes, add 0.5 ml TC 199/20% AB serum medium which contains 0.05 ml PWM solution.

2. Add cell suspension to provide 10 X 10^4 lymphocytes per culture. Amount must be less than 0.5 ml or the tubes will be over filled.

3. Cap cultures and put on rotator in 37°C incubator until time of harvest.

B. Concanavalin A (Con A). Reconstitute contents of vial with 25 ml sterile distilled water. Amount to be used is the equivalent of 10 ug per 1 X 10^6 lymphocytes. Adjust according to size of culture to be set up.

1. Using sterile Fisher tubes, add 0.5 ml TC 199/20% AB serum containing 1.0 ug Con A.

2. Add cell suspension to provide 10 X 10^4 lymphocytes per culture.

3. Cap cultures and put on rotator in 37°C incubator until time of harvest.

C. Unstimulated

1. Using sterile Fisher tubes, add 0.5 ml TC 199 with 20% AB serum.

2. Add cell suspension to provide 10 X 10^4 lymphocytes per culture.

3. Cap cultures and put on rotator in 37°C incubator until time of harvest.

HARVEST PROCEDURE

1. Centrifuge cultures for 2 minutes at 1000 G in the Fisher centrifuge.

2. Remove supernatant, and resuspend cells in buffered saline.

3. Centrifuge at 1000 G for two minutes, and then repeat wash one more time.

4. Resuspend cells in buffered saline to give the desired cell concentration on the slide. The amount of saline may vary dependent on the number of cells present.

5. Apply cells to quartz slide by spreading in a circle approximately the size of the cover slip. Let slides air dry completely.

6. Fix slides in absolute ethanol for 15 minutes. Check slide to avoid evaporation of the alcohol.

7. Let dry and apply quartz cover slip with Zeiss immersion glycerine. Seal with fingernail polish.

APPENDIX

VII. Ultrastructure of Lymphocytes from a Child with Severe Combined Immunodeficiency. 1973. Abstract to be published by the Texas Society for Electron Microscopy.

RASTRUCTURE OF LYMPHOCYTES FROM A CHILD WITH SEVERE COMBINED IMMUNODEFICIENCY.

old Jordan, M.S.[1], B. Sue Criswell*, Ph.D.[2], Mary Ann South*, M.D.[2], and
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dicine[2].

mphocytes from a male infant delivered by C-section and placed into a germfree
vironment have been examined by electron microscopy (EM). The child has a
t-linked severe combined immunodeficiency. At monthly intervals for 9 months,
ml of whole blood from the subject was obtained and the buffy coat fixed for
hour in 3% glutaraldehyde in 0.1M PBS (pH 7.3), post-fixed for 1 hour in 1%
mic tetroxide in 0.1M PBS, and embedded in epon-araldite. Sections were cut
an ultramicrotome, stained with lead citrate and uranyl acetate, and the
mphocytes examined at 80KV. The cells were atypical, having a very sparse
toplasm with little rough endoplasmic reticulum (ER) but abundant smooth ER.
e nuclear membrane was pulled away from the nuclear space, and no evidence
nuclear pores or aggregated ribosomes were found. Mitochondria were intact.
peated injections of KLH and typhoid vaccine, and skin grafting during the
month period yielded no significant observable change in the fine structure
the subject's lymphocytes. At 11 months, the subject was given transfer
ctor, a substance reported to stimulate uncommitted T (thymic) lymphocytes.
llowing repeated injections of this material, the original cell type was
ill present but a new type of lymphocyte was also observed by EM examination.
e new cell type is smaller than normal lymphocytes, has a more dense cytoplasm,
me aggregated ribosomes, detectible amounts of rough ER and more intact nu-
ar membranes. This new type could represent a small population of uncommitted
ells responding to the stimulant (transfer factor). (Supported by NASA
ntract NAS 9-1300, NASA grant NGR 44-003-044, and CRC grant FR-00188 and
A K4-AI-23820.)

APPENDIX

VIII. Changing Populations of Peripheral Blood Lymphocytes in a Gnotobiotic Child with Combined Immune Deficiency. 1973. Abstract to be published by the Leukocyte Culture Conference to be held in Sweden in August, 1973.

CHANGING POPULATIONS OF PERIPHERAL BLOOD LYMPHOCYTES IN A GNOTOBIOTIC CHILD WITH COMBINED IMMUNE DEFICIENCY.

B. Sue Criswell, Stephen L. Kimzey, Mary A. South, and John R. Montgomery. Baylor College of Medicine, Clinical Research Center of Texas Children's Hospital, and Manned Spacecraft Center--NASA, Houston, Texas

A male infant delivered by C-section and placed into a germfree environment has been studied for lymphocytic responses by a number of techniques. The child has a sex-linked severe combined immunodeficiency which was confirmed by initial immunologic studies consisting of PHA, PWM, and antigenic stimulation of peripheral lymphocytes, quantitative immunoglobulins, administration of standard and experimental antigens (KLH) and skin grafting. For the last year the lymphocyte population of this subject has been studied by microspectrophotometry for changes in nuclear and cytoplasmic organization. In three serial studies, peripheral lymphocytes changed following an immunization of the subject with such agents as KLH and typhoid vaccine. Alterations in cellular size and extinction patterns following hematoxylin and eosin staining were detected using a computer-controlled Zeiss Scanning Microscope Photometer. Prior to the immunologic challenge the cells were large with relatively low extinction ranges and resembled immature cell types. Within 4 weeks the cells resembled a more normal population of lymphocytes in size and extinction patterns. These data were also analyzed by a multidimensional clustering computer routine for detection of subpopulations of lymphocytes. Comparisons were made with patterns of cells from normal, control individuals

In contrast to these changes, lymphocytic cultures taken at each of the same sampling periods did not respond to any stimuli with increased uptake of radiolabeled thymidine. Since structural changes in populations of cells were observed, scanning microspectrophotometry coupled with computerized classification of cell types based on extinction patterns appears to be a promising technique for differentiating a new level of response of lymphocytes. It may also yield useful information concerning the level of defect in a non-functional population of cells.