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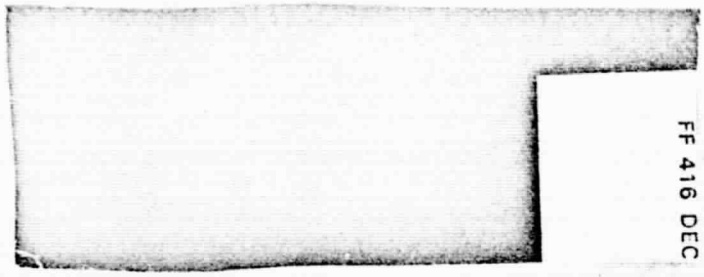

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FINAL REPORT

Contract NAS 9-8918

BIOLOGICAL ACTIVITY OF LUNAR SOIL

January 7, 1970 - October 31, 1970

Prepared by

Alan J. Kenyon, D.V.M., Ph.D.
Professor of Biochemistry
Department of Animal Diseases
University of Connecticut
Storrs, Connecticut 06268

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I. ABSTRACT OF RESEARCH ACCOMPLISHMENTS

During the contract period of January 7, 1970 through October 31, 1970, clinical pathology procedures for the detection of diseases in germfree mice maintained within Class III isolators were further developed and tested. The information obtained was supplied in detailed monthly Progress Reports to NASA.

In transmission experiments, murine hepatitis virus (MHV_3) has been shown to infect CD-1 mice (previously not demonstrated in this strain). At 2 to 4 days postinoculation the spleen weights were reduced and had lymphocytic karyorrhexis in the thymus-dependent area of splenic follicles. The germinal center assay was found to be a superior indicator of immunologic stimulation as opposed to the classic complement fixation procedures. At 6 to 8 days postinoculation, concomitant with an increase in spleen weight, the germinal centers were near maximum in size and number. Complement fixation titers revealed antibody to MHV_3 at 16 to 32 days postinoculation. Like the lactic dehydrogenase virus (LDV),* MHV_3 inhibits cellular immunity as measured by skin graft rejection. In both LDV and MHV_3, the loss of cellular immunity was associated with lymphocytic karyorrhexis of the mobile population of small lymphocytes. An assay method has been developed for MHV_3 using LDH levels as an indicator of infection rather than histologic examination.

Macrophage cultures will support MHV_3 irrespective of the age of macrophage donor mice.

Thymosin, which induces a heightened germinal center response, shows no detectable histologic alteration in the thymus-dependent area of the splenic follicle even though it has been shown to accelerate graft rejection (enhanced cellular immunity).

LDV has been passaged and used as another test system for enzyme assays. LDV causes an increase in LDH at 2 days postinoculation which is maintained throughout the life of the infected mouse. LDV inoculates show a marked increase in spleen weight at 16 days postinoculation.

Plasmodium berghei (the agent of murine malaria)** has been passaged in both mice and hamsters. In mice, P. berghei results in a decrease in body weight, and a concurrent absolute decrease in liver and kidney weights, while spleen weights become increased by 8 days postinoculation. There is a moderate increase in serum LDH at 4 hours postinoculation, which

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*Dr. A. L. Notkins, National Institute of Dental Health, N.I.H.

**Dr. S. Criswell, NASA, MSC, Houston, Texas

returns to normal before showing a severe increase by 6 days postinoculation. In hamsters, although the body weight remained constant, there was a 6-fold increase in spleen weights by 8 days postinoculation. Serum LDH levels were moderately increased at 5 days postinoculation. There was a 15 to 25% decrease in hematocrit by 8 days postinoculation which paralleled the number of parasitized red blood cells. In parasitized mice, the increase in LDH values corresponded to increased hematopoiesis in spleens. Similarly, in hamsters the amount of hematopoiesis in spleens was relatively low as was the response in serum LDH, indicating the origin in mice may be related to increased activity rather than cellular destruction.

Design and construction of an aerosolization chamber for the study of titanium-containing dusts has been completed. Standard curves have been obtained spectrophotometrically with TiO_2. Titanium has been detected in the lymphoid structures of hamsters, mice, and rats. In a prototype chamber, rats were treated with aerosolized TiO_2 and distribution of titanium established.

II. LIST OF MANUSCRIPTS AND PUBLICATIONS SUPPORTED IN PART BY CONTRACT NAS 9-8918

A. Adams, M. L., Kenyon, A. J., Jones, N. D., Schmidt, N. F., and Kim, S. N. Lymphoproliferative diseases of fowl - LDH isozymes associated with lymphoblastic leukemia (JM-V). J. Natl. Cancer Inst. 46: 43-48, 1971.

B. Olmsted, J., and Kenyon, A. J. Lymphoproliferative diseases of fowl - Radiologic evidence of transplantable nature of avian lymphoblastic leukemia (JM-V). Submitted to Avian Diseases.

C. Kenyon, A. J., Smith, G. V., Kim, S. N., and Fredrickson, T. N. Depressed cellular immunity and alterations of thymus-dependent areas associated with virus replication in macrophages. Submitted to Nature.

D. Olmsted, J., and Kenyon, A. J. Lymphoproliferative diseases of fowl - Lymphoblastic leukemia (JM-V) as a radiologic model. Submitted to Acta Radiologica.

E. Kenyon, A. J., and Jones, N. D. Dysproteinemia of Coturnix quail associated with ova reabsorption. For submission to Avian Diseases.

F. Kenyon, A. J., Olsted, J., Fredrickson, T. N., and Jones, N. D. Correlation of germinal center response with development of lesions in gnotobiotic mice inoculated with murine hepatitis virus. For submission to the American Journal of Veterinary Research.

G. Adams, M. L., Kenyon, A. J., Olsted, J., LaDoux, R., and Jones, N. D. Early detection of murine malaria (Plasmodium berghei) by LDH isozymes. For submission to the American Journal of Veterinary Research.

III. PARTICIPANTS

A. Principal Investigator

A. J. Kenyon, D.V.M., Ph.D.
Professor of Biochemistry

B. Research Assistant II

Mary Adams, B.S.
Judy Olsted, B.A.
Richard Boyden, B.A.

C. Student Labor

David Scott
Judith King

D. Collaborating Researchers

T. N. Fredrickson, D.V.M., Ph.D.
Associate Professor

G. Smith, M.D.
Assistant Professor

S. N. Kim, D.V.M., Ph.D.
Associate Professor

D. S. Wyand, D.V.M.
Associate Professor

IV. BUDGET DESCRIPTION

Detailed budget report will be prepared by the Office of Research Accountant, Mr. H. Dest, University of Connecticut, Storrs and submitted seperately.

A. Salaries and Wages

1. A. J. Kenyon, Principal Investigator 10% Academic Year (9 months)	None
2. S. N. Kim, Co-Principal Investigator 10% Academic Year (9 months)	None
3. Mary Adams, Research Assistant II 100% Academic Year (12 months)	$7200
4. Graduate Assistant (M.S., 12 months)	4000
5. Student Labor @ $2.00/hour	2000
TOTAL SALARIES AND WAGES	$13,200

B. Applicable Fringe Benefits @ 8%	1,056
C. Indirect Costs (53% of Salaries and Wages)	6,996
D. Supplies	2,340

1. Reagents

a. immunological	$200
b. enzyme substrates	200
c. chemicals	200
d. isotopes, immuno-tests	250

2. Animals

a. mice (CD-1), 200 germfree @ $4.00 each	$800
b. feed (germfree) Model 25 shipper and 25 lb. feed $50	100
c. mice (CD-1), 200 conventional @ 45¢ each	90
d. quail eggs, 400 @ 25¢ each	100
e. quail chicks, 100 @ 4.00 each	400

E. Equipment 2,500

1. Electronic components for modification of existing electrophoretic equipment $500
2. Incidental equipment of cell fractionations 200
3. Isolators:

Germfree Associates (rigid)	1,000
Smyder (flexible film, 2 @ $400 each)	800

F.	Travel		$1,000
	1. Three trips to MSC/NASA	$750	
	2. Transportation to pick up supplies and animals	250	
G.	Other		
	1. Publications Costs		200
		TOTAL REQUESTED	$27,292

V. DETAILED REPORT

A. Overall Objectives

The objectives as described here represent an organizational modification of the STATEMENT OF WORK (See Appendix I for details).

1. Develop and test procedures for biochemical interpretation of disease.

2. Gain base-line information on the immunolgoc responsiveness of CD-1 germfree mice (Charles River Breeding Laboratory).

3. Evaluate the germinal center assay for detection of antigenic agents by infecting CD-1 mice with known viruses and protozoan organisms.

4. Evaluate thymosin for its ability to heighten germinal center response to standard antigens.

B. Research Accomplishments

1. Studies on MHV_3 as a Test System for Procedures Used in Class III Cabinetry.

The murine agent MHV_3 was selected as one of a series of test agents because in mature mice it induces only a transient disease with extremely low mortality, yet the disease may be easily monitored by procedures described in the test protocol for sample analyses (enzyme assay; lactic dehydrogenase [LDH]). The MHV_3 agent has been passed several times in CD-1 mice (COBS) since originally obtained from Dr. T. N. Fredrickson, Department of Animal Diseases, University of Connecticut. The original passage was from 7-day-old BALB/c pups. The infectivity of the inoculum has been greatly increased by harvesting livers of 10- to 14-day-old CD-1 pups on the 4th to 5th day postinoculation rather than waiting until massive liver lesions occur, as is commonly done.

To determine the peak infectivity (maximum yield of agent), pups 10 to 14 days old, were inoculated at killed at 4 and 6 days post-inoculation. In addition to harvesting tissue, blood was obtained for serum LDH assay. The release of LDH appeared to correlate well with progressive destruction of macrophages and hepatocytes. When necrotic foci were apparent on gross postmortem, the enzyme levels were declining to normal values which indicates that destruction of cells had already occurred and that repair processes were taking place. Figure 1 diagramatically shows the relationship of events associated with MHV_3 infection.

a. Histologic evaluation

In each of the transmission experiments, 24- to 28-day-old germfree CD-1 mice were housed in Snyder flexible film isolators. From 48 to 96 hours postinoculation, MHV_3 inoculates

developed large areas of necrosis (lymphocytic karyorrhexis) in the "thymus-dependent areas" of splenic follicles. In many follicles the cellularity surrounding the central arteriole was totally obliterated. In addition to the focal necrosis observed in livers of MHV_3 infected mice, death of macrophages and lymphocytes in the "thymus-dependent area" of splenic lymphoid follicles was a consistent observation at 48 hours postinoculation. This lesion is less evident at 4 days postinoculation and is not apparent at 8 days post-inoculation (Table 1).

Figure 2 shows the nature of lesions seen in spleens of mice inoculated with LDV, MHV_3, and the MHV_3 agent in combination with the Aleutian disease virus.

b. Spleen weight increase

A consistent finding was that the spleens of MHV_3 inoculates either dropped in weight or remained unchanged during the first 4 days postinoculation. After this depression in weight, the MHV_3 inoculates developed a rapid increase in spleen weight (200-240 mg.) at day 8 postinoculation. This increase in weight appeared to be due to an increase of inflammatory cells in the splenic red pulp (Figure 3).

c. LDH increase

Because MHV_3 may not produce death in test mice, a test system was devised whereby 10-day-old CD-1 pups were used as an assay subject with infection being established on the basis of elevated LDH levels at 6 days postinoculation. This procedure was more sensitive than histologic analyses and much less time consuming and costly (Figure 4).

d. Complement fixation

Complement-fixing antibodies were sought by titration to MHV_3 by Microbiological Associates, Bethesda, Maryland. Levels of antibody were detectable at 16 to 32 days post-inoculation, but it was apparent that the germinal center assay was a better indicator of immunologic stimulation since it preceded detectable serum antibody levels by at least 10 days.

e. Graft rejection

The effect of MHV_3 infection on cellular immunity was determined using skin allograft rejection. Standard, full-thickness, skin grafts were made from C57BL/6 male donors to 21-day-old CD-1 male recipients. There was a delay in graft rejection of 2 to 3 days in MHV_3 infected mice. Since the thymus-dependent area of the spleen is associated with cellular immunity, the inhibition of immune response may be the result of the destruction of cells in the "thymus-dependent area" of the spleen. Similar damage induced by release of

proteolytic enzymes from macrophages may account for destruction of hepatocytes and also contribute to altered immunity. Figure 5 shows the gross appearance of skin grafts on infected and normal mice.

f. Peritoneal macrophage culture

Macrophage cultures were established to test for and maintain MHV_3 passage inocula. Murine macrophages were obtained by repeated intraperitoneal inoculations of tetanus toxoid. Peritoneal exudate cells were washed out with medium 199. Estimates of cell populations are made by cell counting on a hemocytometer. Cells were then placed in Leighton tubes, gassed, and incubated. After 72 hours incubation the macrophages are usually well differentiated and ready for inoculation with a 1:10 dilution of 10% liver homogenate from MHV_3 infected mice. The inoculated cultures were incubated for 48 hours. The susceptibility of different stains of mouse macrophages was as follows: LAF / A/HE / C57BL/6 as determined by morphologic changes and cell death at approximately 12-hour intervals from time of inoculation. At 48 house postinoculation the degree of destruction was so advanced, no detectable difference between cell populations was obtainable.

2. Studies on Thymosin for Immunologic Enhancement

Thymosin prepared from fetal calf thymus induces a heightened germinal center response to heterologous erythrocytes used as antigens. A calf kidney extract compared on an equal weight basis induced very little germinal center response.

The experiments performed with thymosin show the consistent effect it has on reducing the rejection time of allografts made from C57BL/6 to CD-1 recipients. The effect of MHV_3 in delaying rejection is an equally consistent factor. See Figure 7 for graft rejection rates of mice administered thymosin and infected with MHV_3.

Although as anticipated, the main effect of thymosin is not on germinal center formation but rather is on the mobilization of small lymphocytes in the thymus-dependent areas of splenic follicles. However, there was indeed an increased rate of germinal center formation in stimulated mice.

3. LDV as a Test System for Class III Cabinetry

The LDV agent was administered by the intraperitoneal route to 16 germfree 23-day-old CD-1 mice, to be killed at 2, 4, 8, and 16 days postinoculation. Organ weights were recorded. Serum was obtained for LDH assay and tissues were obtained for histologic observation.

a. Spleen weight increase

At 2 and 4 days postinoculation spleen weights of control animals were greater for uninoculated controls, however, by day 8, LDV inoculates showed a marked increase which was maintained to day 16 (Figure 8).

b. LDH increase

LDV inoculates showed a 2-fold increase in LDH at 2 days postinoculation, and a 3-fold increase by 4 days. In some instances, a transient drop occurred at 8 days with levels increased 3 to 4-fold again by 16 days postinoculation. The range of LDH values as spectrophotometric units was from 3,800 to 10,000 during the course of disease. Mice infected with LDV never return to normal serum levels throughout their life (Figure 9).

c. Histologic evaluation

The only detectable histologic lesion in LDV infected mice occurs at 48 hours postinoculation. This lesion is lymphocytic karyorrhexis of the thymus dependent area of splenic follicles. Similar lesions are seen in mice inoculated with MHV_3 and the lymphocytic choriomengitis virus. It is tempting to speculate that virus replication in macrophages results in release of proteolytic enzymes causing destruction of associated cellular structures. See Figure 2 for a comparison of lesions of LDV and MHV_3.

4. Studies with Protozoan Agents (Plasmodium berghei)

To extend the spectrum of agents previously used as test systems to evaluate the analytical procedures conducted on mice in Class III cabinetry, murine malaria was selected as a protozoan agent capable of causing an acute response.

Plasmodium berghei has been serially passaged 18 times during the course of this study. The original inocula was passed 3 times in mice before transmission to hamsters. Ten passes were made in hamsters prior to the disease being passed back into mice and carried 5 passages. In each case, parasitized red blood cells were obtained by bleeding from the supra-orbital plexus. Per cent parasitized cells were estimated, in each case, by observations made on blood smears stained with Wright-Giemsa (Figure 10).

a. Mouse experiments

Mice inoculated with Plasmodium berghei show a decrease in body weight, and an absolute decrease in liver and kidney weights which is in accord with the decrease in body weights. The spleen weights of inoculates, however, show an absolute increase which was evident aa early as 4 days postinoculation (Figure 11).

Serum LDH levels of Plasmodium berghei inoculated mice show a rapid increase to 6,000 units at 4 hours postinoculation as an early response. A marked increase to 27,000 units occurred at 6 days postinoculation as a late response (Figure 12).

Early in the course of the disease (2 days postinoculation) the principal splenic lesion may be related to the large number of histiocytes in the red and white pulp. As the disease progresses, evidence of increased hematopoiesis is apparent. By 4 days postinoculation hemosiderin deposits are apparent throughout the liver parenchyma. From this point, the main lesion consists of hyperplasia of the red pulp and progresses to such an extent that it destroys the normal splenic architecture.

b. Hamster experiments

Hamsters inoculated with Plasmodium berghei show no decrease in body weight, but do exhibit a 6-fold increase in spleen weights which is detectable 4 days postinoculation (Figure 13).

Plasmodium berghei hamsters show only a moderate increase in serum LDH activity to 5,000 units at 5 days postinoculation (Figure 14).

The distribution of lesions in hamsters includes inflammatory cells in liver and kidney as perivascular cuffs (at 8 days postinoculation). Although the liver contains considerable hemosiderin, the spleen does not have the tremendous hyperplasia of the red pulp as is seen in mice. The bone marrow of inoculated hamsters consisted mainly of blast forms with many cells in mitosis.

As in mice, hamsters inoculated with Plasmodium berghei show a marked increase in per cent parasitized red blood cells to almost 100% at 6 days postinoculation (Figure 15). As may be expected, the hematocrit decreases from 50% to 25% at 8 days postinoculation (Figure 16).

5. Preliminary Studies on the Biologic Effects of Inhaled Lunar Dust

a. Aerosolization chamber

Construction of a chamber for the housing of germfree or conventional animals to be used in inhalation studies with simulated lunar dust, titanium and silicon dioxides, has been completed (working drawings to be submitted in future reports).

b. Studies with titanium dioxide: standard curves

The procedure of Huggins and Froehlick (J. Exptl. Med. 124: 1099, 1966) has been modified and applied to titanium dioxide, obtained from Dupont as Ti-pure R-900, to yield linear curves

with a range of 10 to 1,000 ug. This procedure has been applied to detection of TiO_2 from lymphoid structures of hamsters.

c. Experimental results

In a modified aerosolization chamber, 23-day-old male rats were exposed to aerosolized titanium dioxide. Animals were killed at 6, 12, and 24 hours postinoculation. Another 3 animals were inoculated intravenously (tail vein) with 0.8 ml. of 250 mg. TiO_2/ml. saline. Histologic evaluation of tissues revealed titanium dioxide in alveolar macrophages and as time progressed in the peripheral zone of splenic follicles (Figure 5).

VI. APPENDIX

A. Statement of Work

1. Purpose

To obtain workable methods for testing biologic activity of lunar soil in and on homoiothermic subhuman animals. In order to do this, the techniques must be capable of differentiating physical (toxic) activity from biologic (antigenic) activity.

2. Objectives, General

a. Develop standard operating procedures (SOP's) for toxicologic and immunologic techniques.

b. Verify these technisues with known toxic and immunogenic material.

c. Supply results and interpretation of these tests.

3. Objectives, Specific

a. Although the techniques may be worked out on any mice or quail, the SOP's and results must be directly applicable to those strains used by the Lunar Receiving Laboratory (LRL). The animals used are germfree and conventional random-bred Charles River CD-1* strain of mice and the Coturnix coturnix japonica strain of quail.

b. The SOP's and results for the mice must take precedence although the work may proceed concurrently with both species.

c. All SOP's must take into consideration the constraints of of Sample Laboratory of the LRL, i.e., space, personnel, and Class III quarantine cabinetry.

d. Evidence of antigenic stimulation will be sought by evaluation of host reaction in lymphoid structures in response to soluble antigens.

e. Response to particulate antigens (lunar or simulated lunar material) by a number of antigen-antibody reactions will be tested.

f. Infectivity following confirmed antigenic response will be obtained by quantitative estimates of response in successive passages of the material.

g. Immunochemical and electrophoretic methods will be used to show evidence of dysproteinemias of clinical significance to the various biologic and nonbiologic materials.

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*The Charles River Breeding Laboratories, Inc., Wilmington, Mass. 01887.

B. Work Plan for Future Studies

1. The contractor shall initiate a pilot program to determine the immunologic, physiologic, and morphologic changes induced with aerosolized simulated lunar dusts.

2. The quantitative distribution of dust shall be determined in lymphoid and other tissues of mice and ferrets given dust by different modes (insufflation, inhalation, intravenously, and intraperitoneally).

3. The effect of macrophage depletion with titanium and dilicon dioxides and SLD shall be determined initially in relation to several infectious agents which produce either an acute or chronic disease.

4. The acute response shall be determined in ferrets aerosolized with SLD and challenged with aerosolized influenza virus (influenza A PR8). These experiments shall be constructed to yield an index of pathogenicity or disease enhancement as a function of dust exposure.

5. The effect of TiO_2, SiO_2, and SLD on chronic or slow disease processes shall be determined in ferrets aerosolized with the Aleutian disease virus. The experimental design shall be constructed to relate dust exposure to time required for primary onset of lesions.

6. Mice (DBA_2) shall be aerosolized with SLD and TiO_2 to determine the enhancement of latent leukemogenic viruses. Experiments shall be constructed to determine the effect dust has on induction of parenteral and inhalation leukemogenesis and on the reaction of peripheral and central lymphoid organs in response to virus (Friend virus) induced lymphoproliferation.

7. Determine the effect specific metals or metal complexes (analogous to lunar dust) have on germinal center formation and deposition in lymphoid tissue.

Figure 1. MHV_2-Response Relative to Infectivity

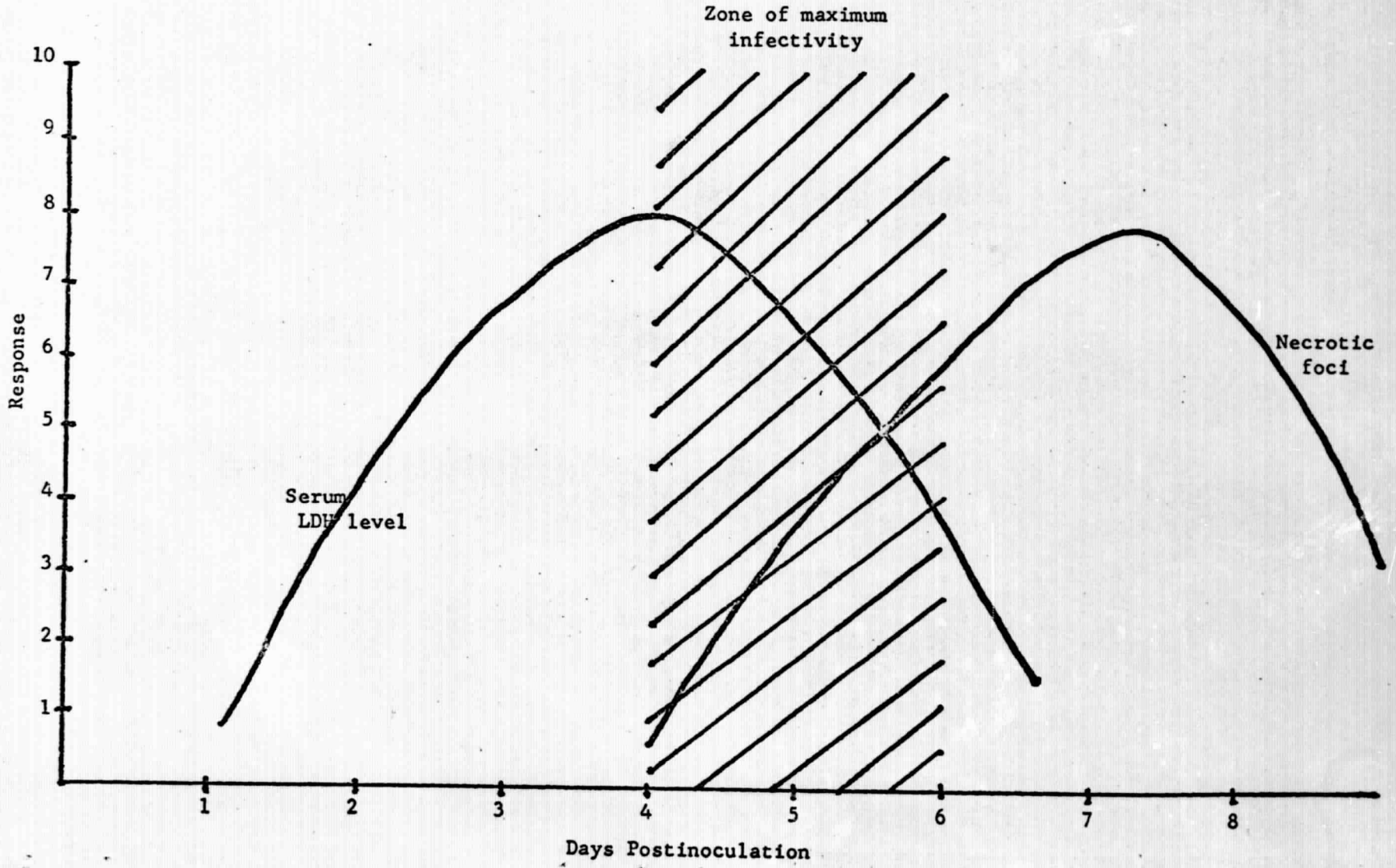
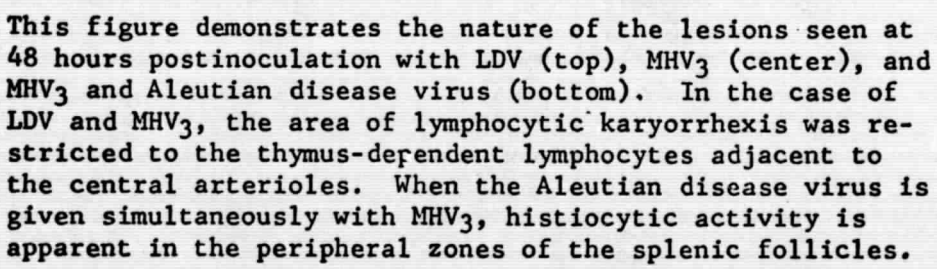


Figure 2.



This figure demonstrates the nature of the lesions seen at 48 hours postinoculation with LDV (top), MHV_3 (center), and MHV_3 and Aleutian disease virus (bottom). In the case of LDV and MHV_3, the area of lymphocytic karyorrhexis was restricted to the thymus-dependent lymphocytes adjacent to the central arterioles. When the Aleutian disease virus is given simultaneously with MHV_3, histiocytic activity is apparent in the peripheral zones of the splenic follicles.

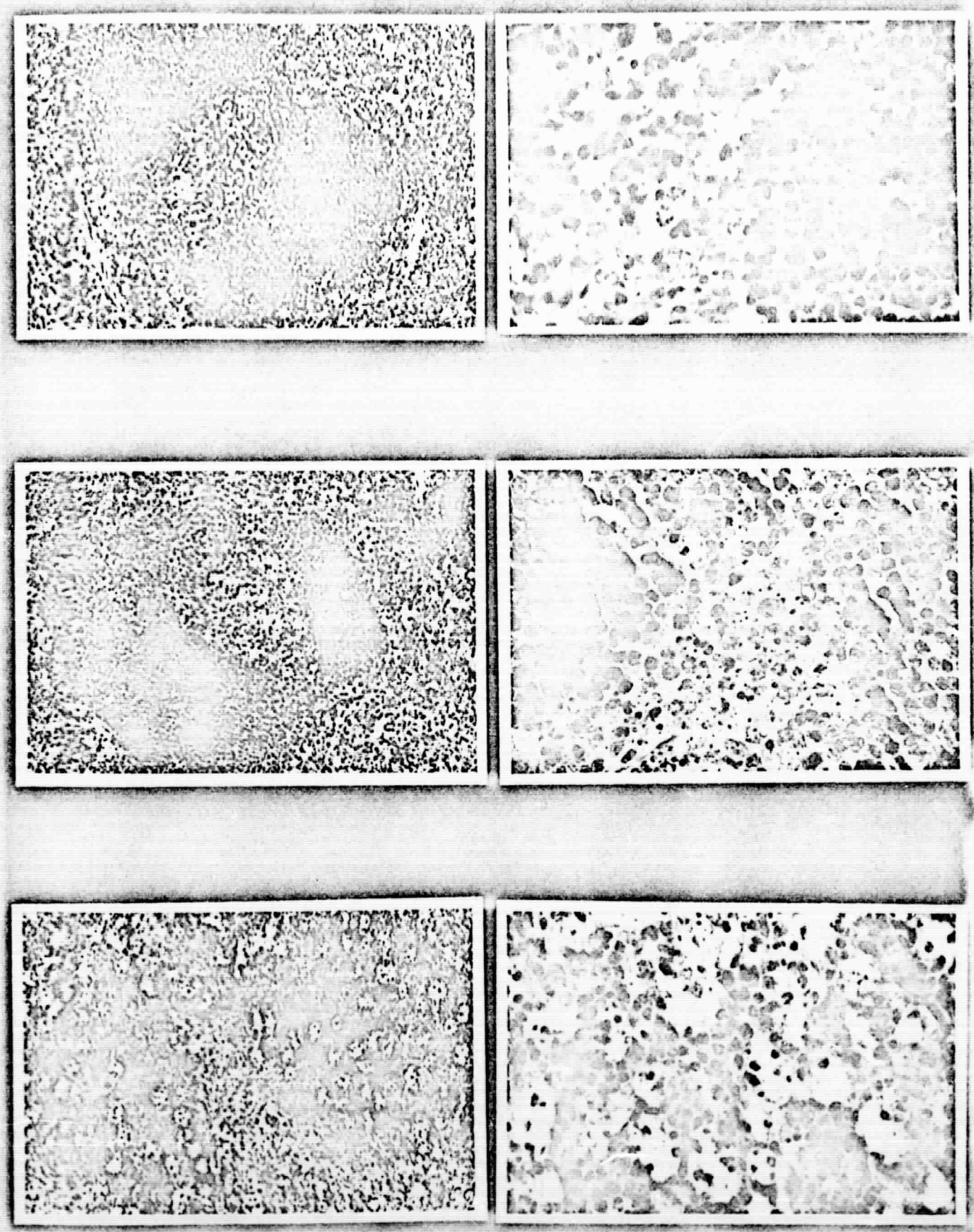


Figure 3. MHV_3 - Spleen Weight

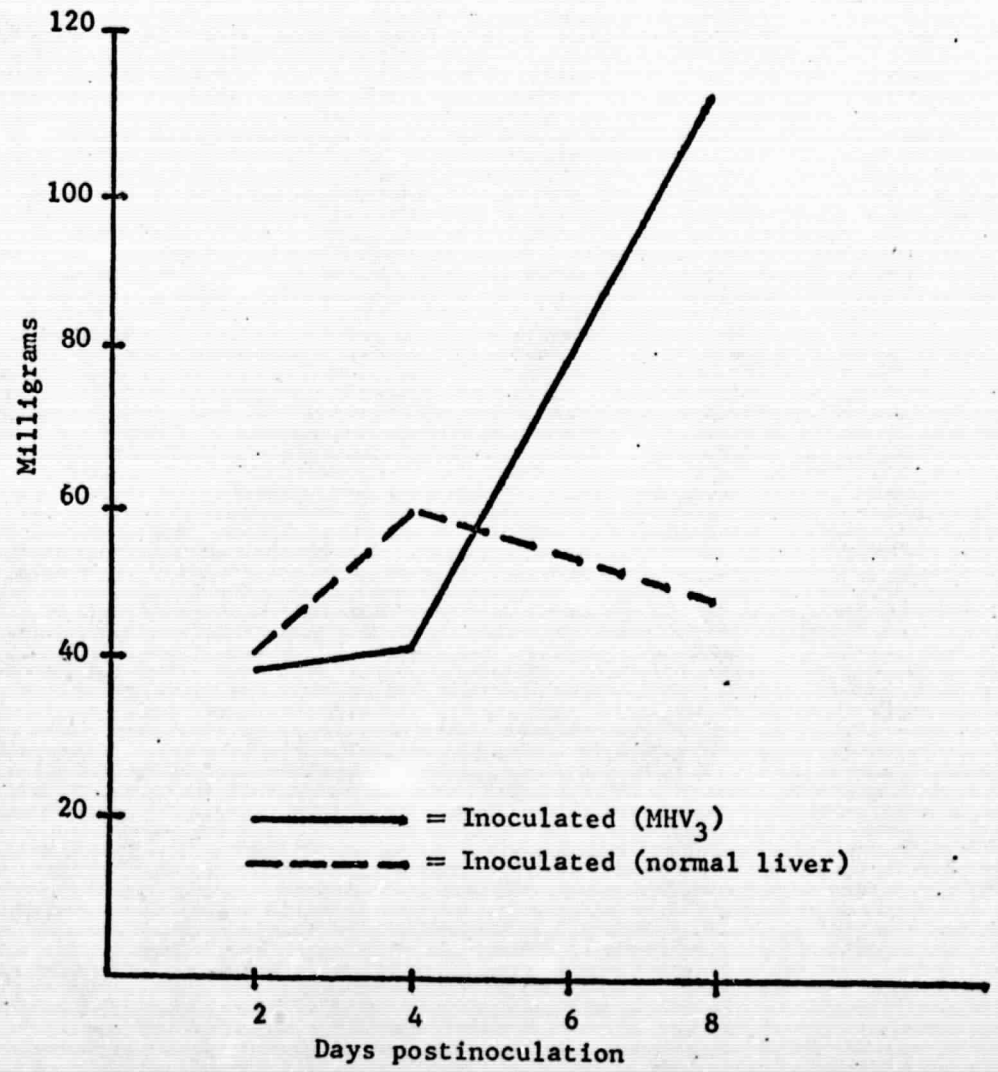


Figure 4 MHV_3 - LDH

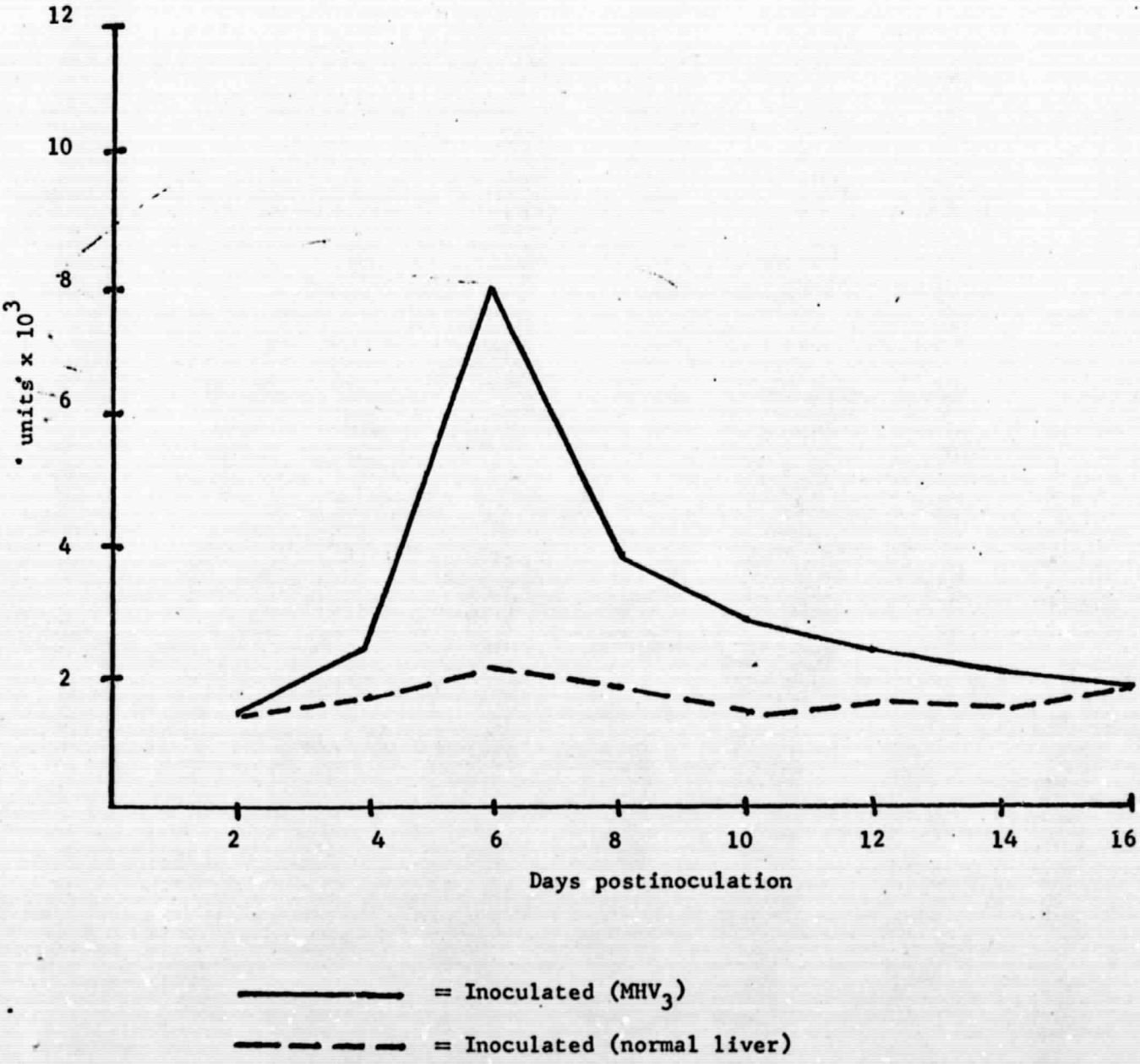
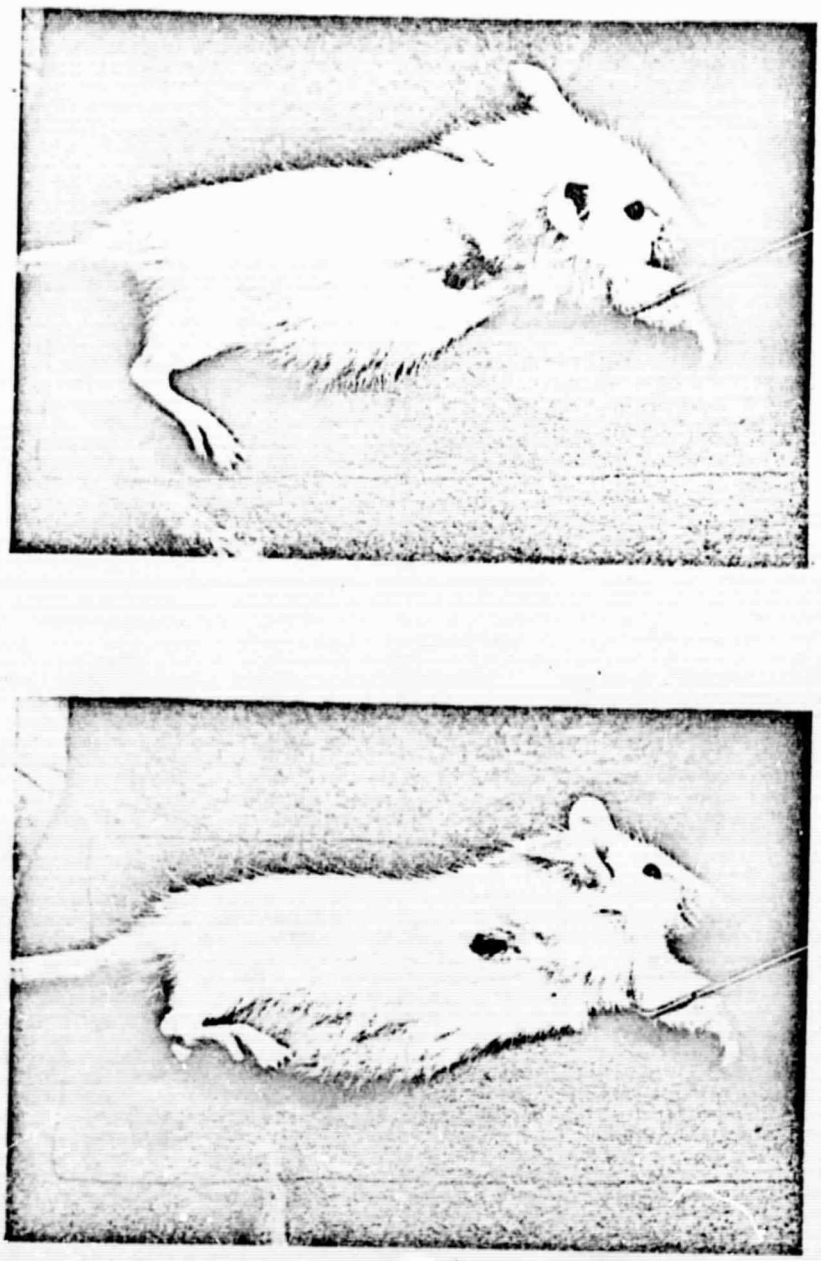
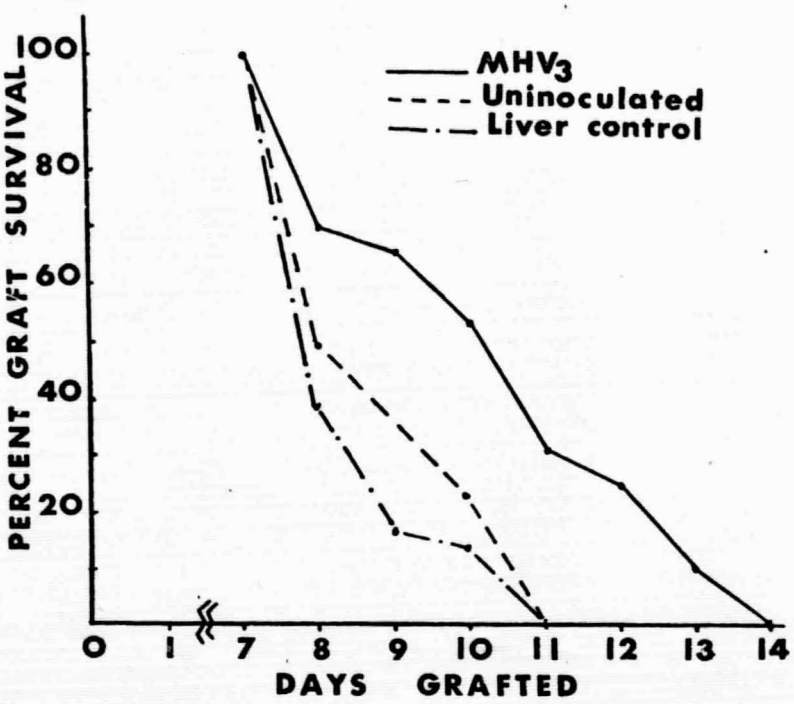


Figure 5.



Photographs taken at 8 days after transplantation of skin from C57/BL donors to CD-1 recipients. The mouse in the upper photograph was infected with the MHV_3 virus and, as may be seen, its graft appears healthy. The mouse in the lower photograph is an uninoculated control and its graft is in an advanced state of rejection.

Figure 6.



This photograph depicts the rate of rejection of MHV_3 infected mice with skin grafts.

Figure 7. Per Cent Survival of Skin Grafts

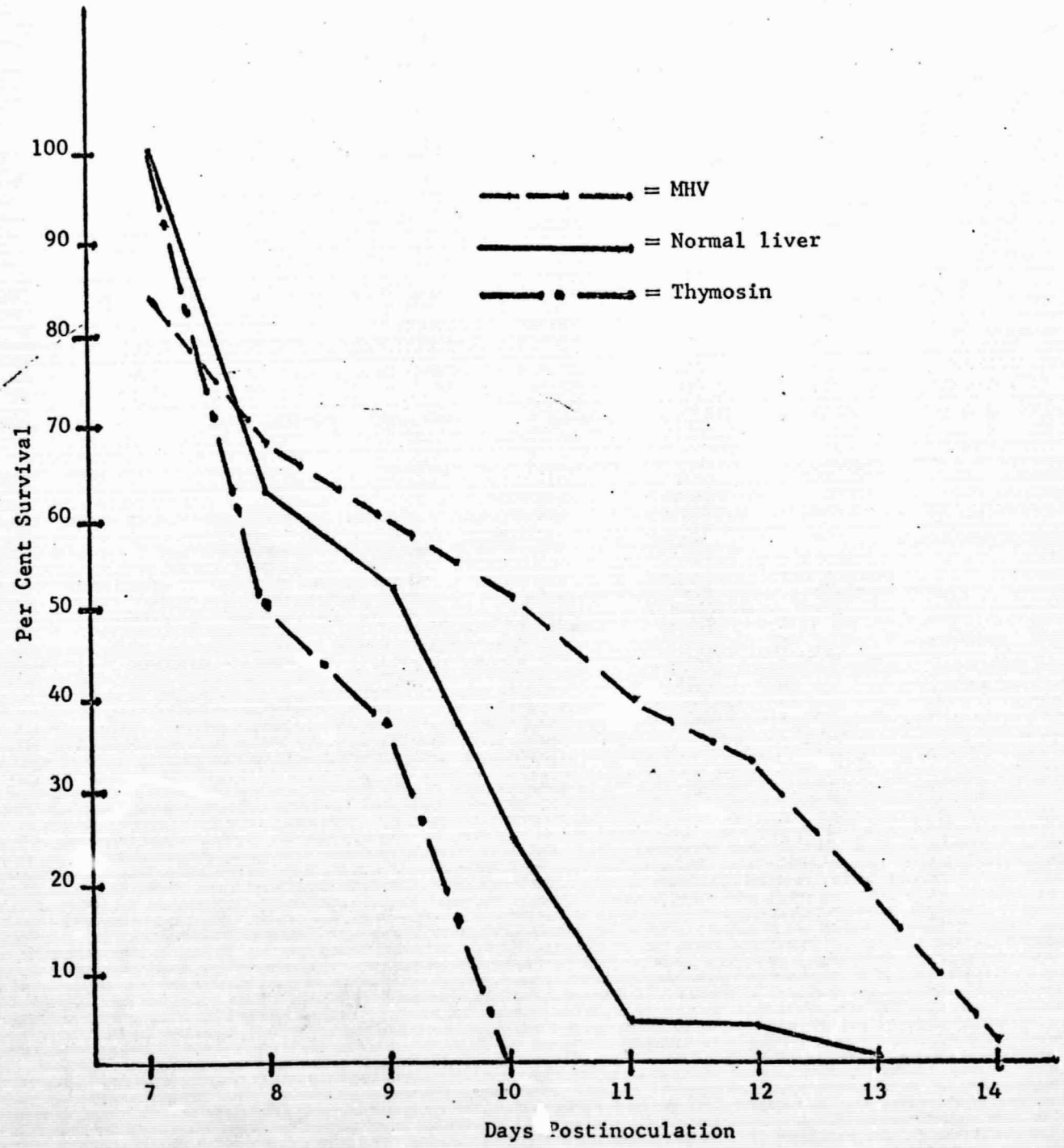


Figure 8. LDV - Spleen Weight

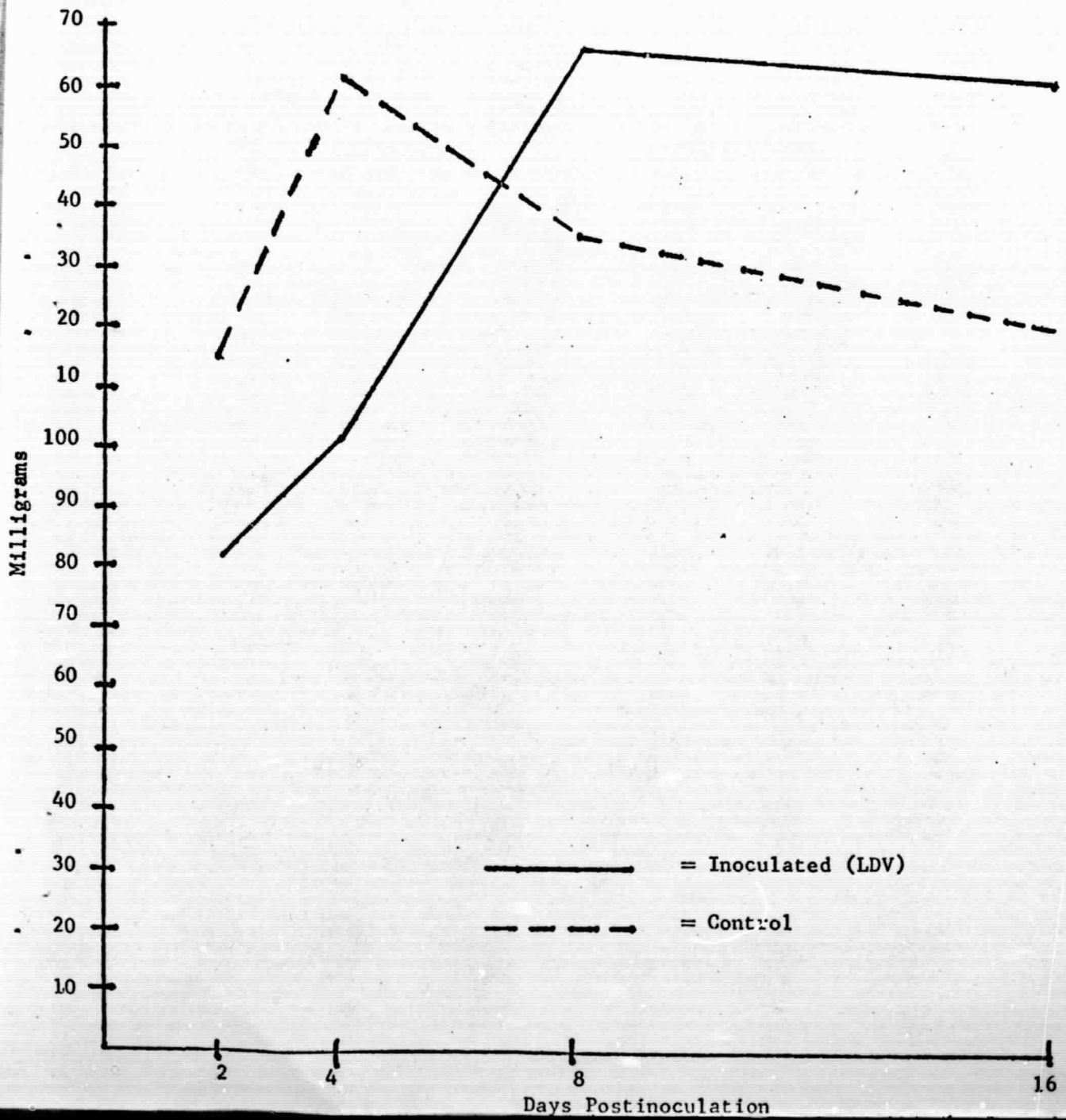


Figure 9. LDV - LDH

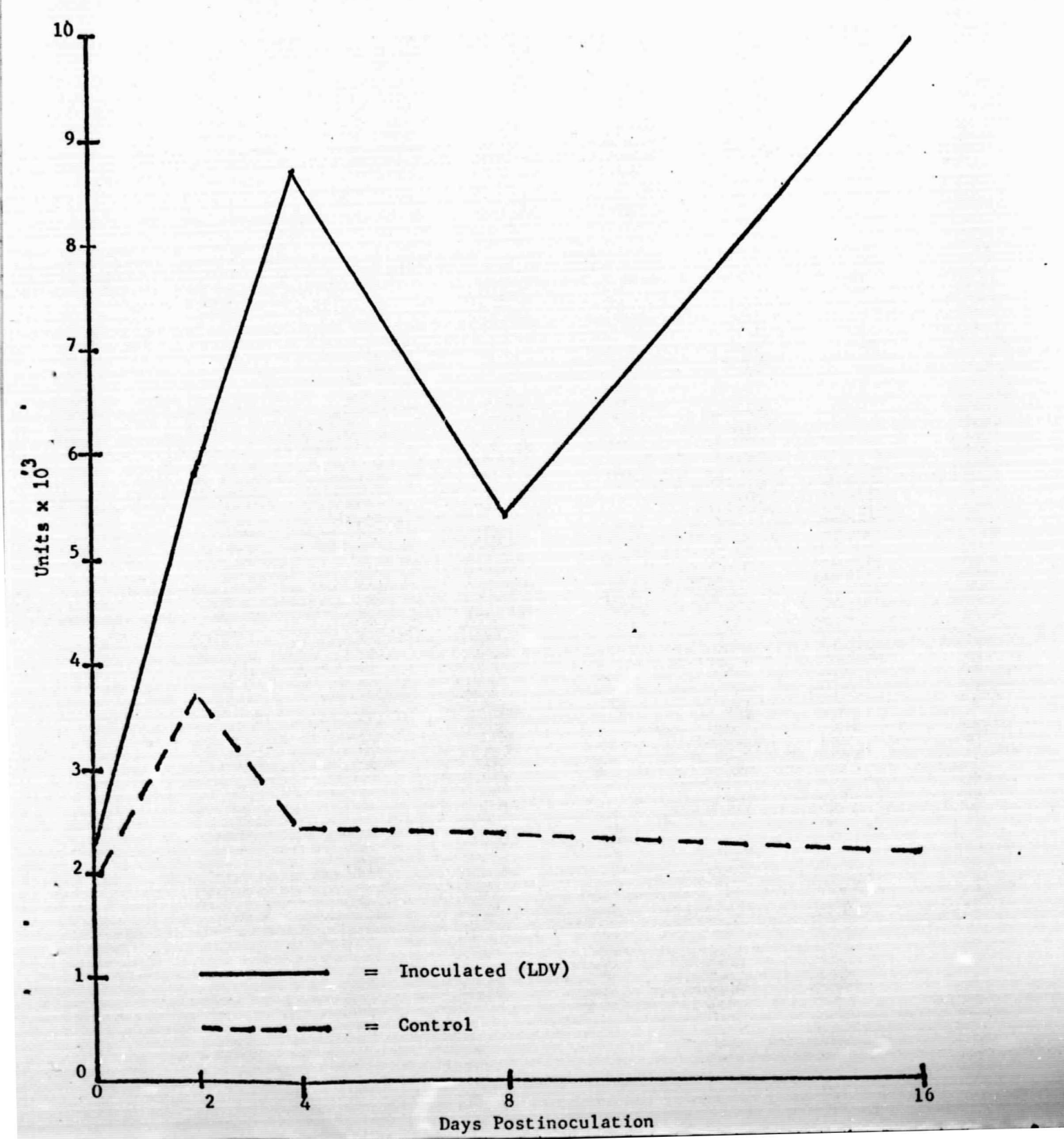


Figure 10.

This photograph demonstrates the extensive parasitemia that existed in mice 5 days postinoculation with Plasmodium berghei. At this stage the LDH values have reached a peak and will slowly decrease.

Figure 11. Plasmodium berghei in Mice - Spleen Weights

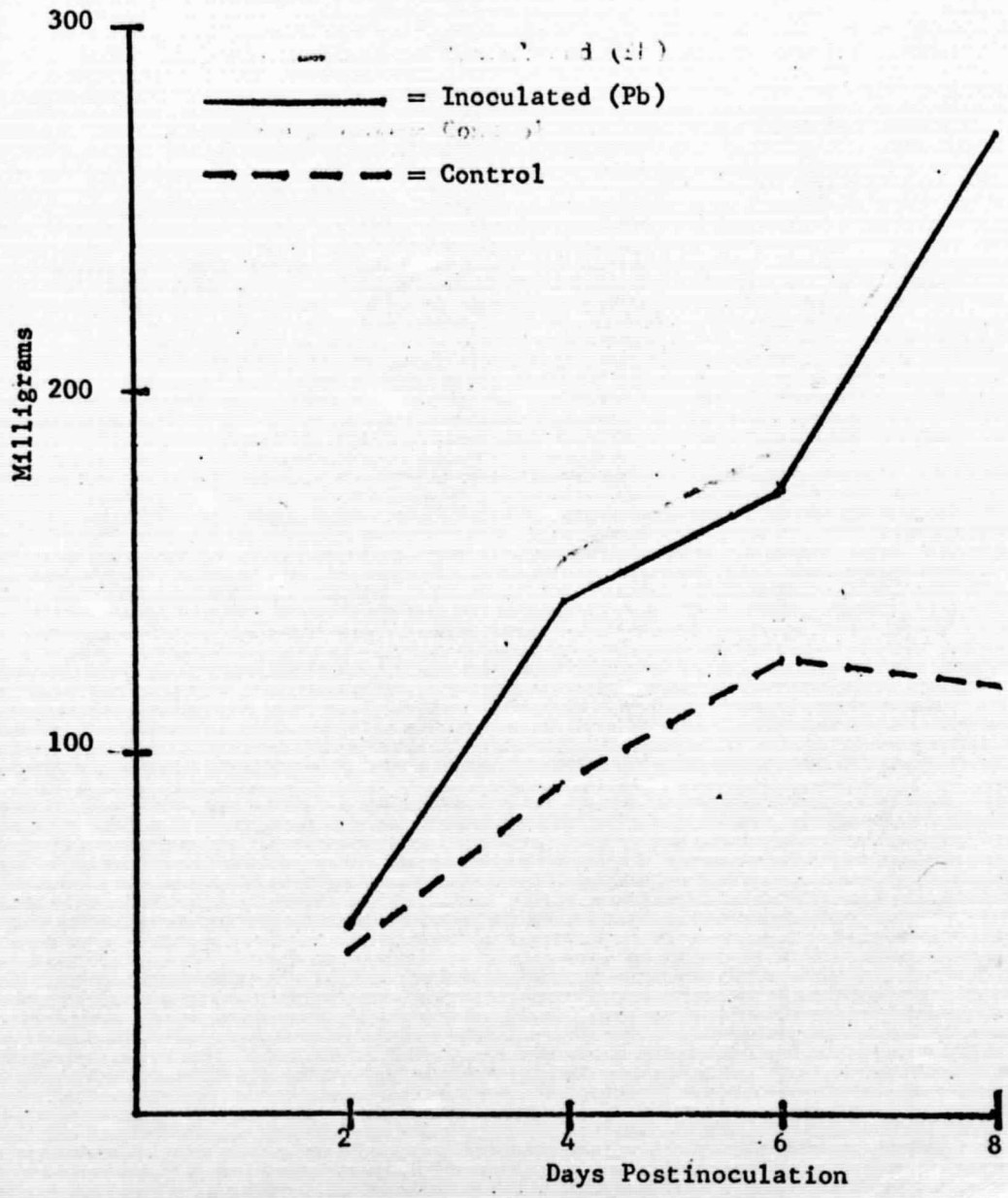
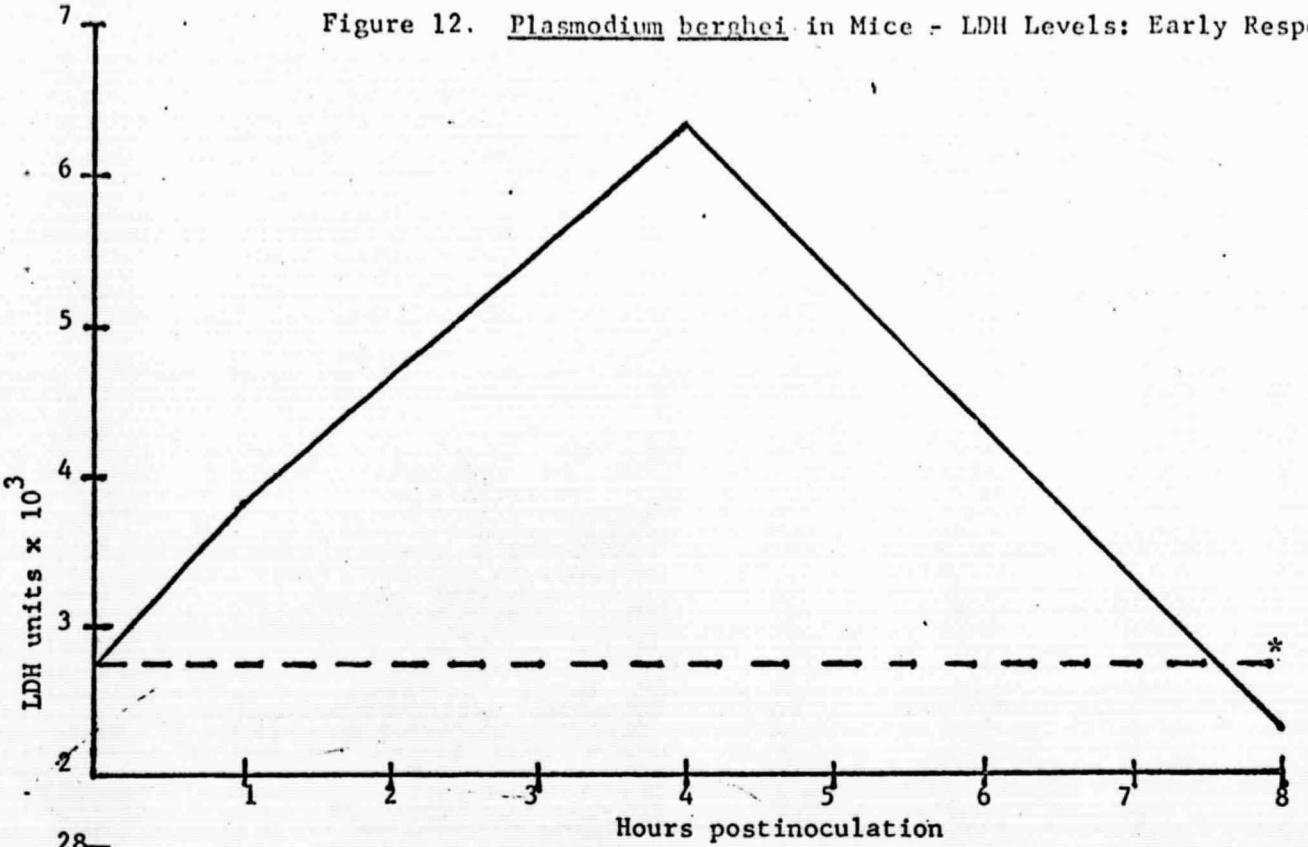


Figure 12. Plasmodium berghei in Mice - LDH Levels: Early Response



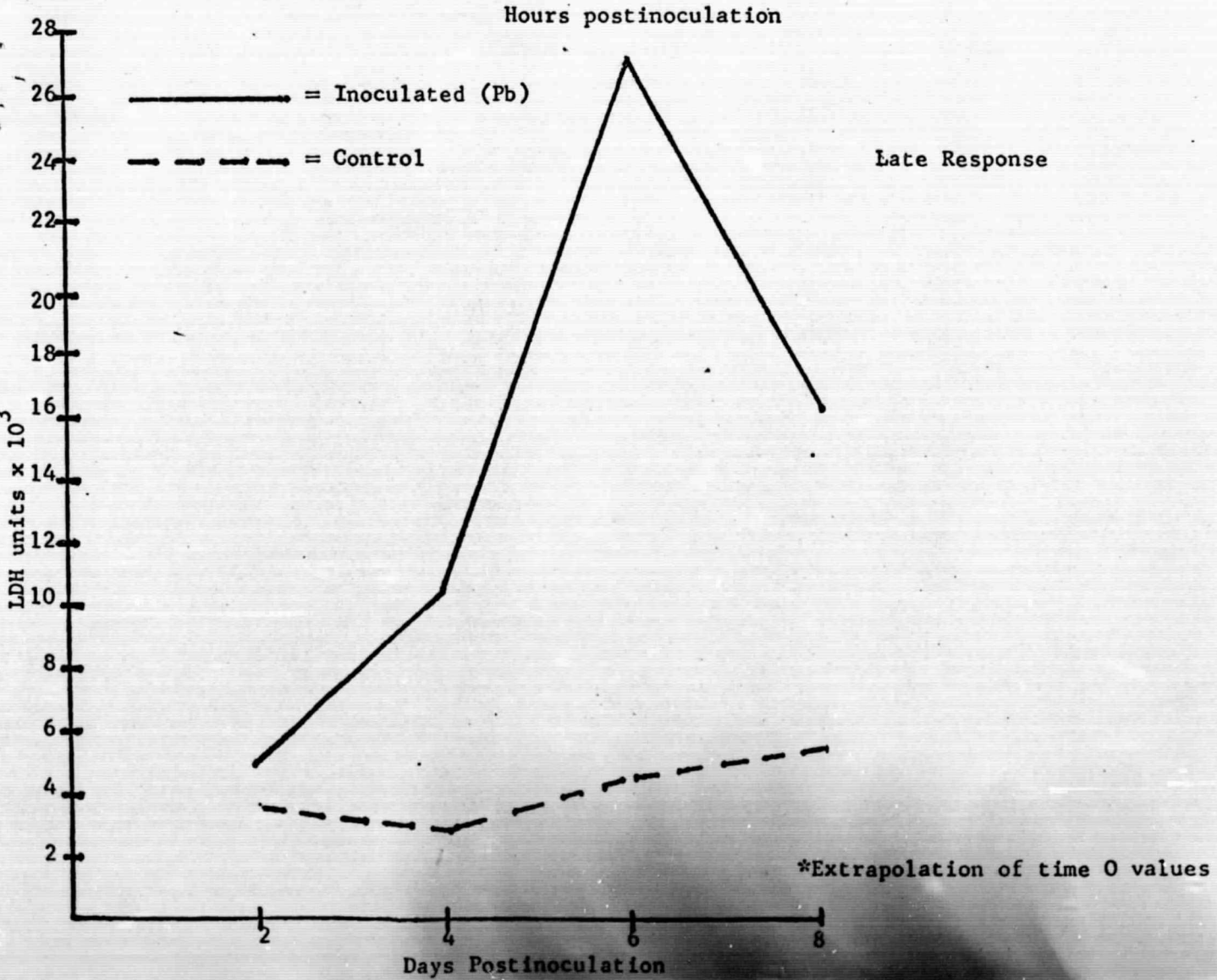


Figure 13. *Plasmodium Berghei* in Hamsters

Spleen Weights

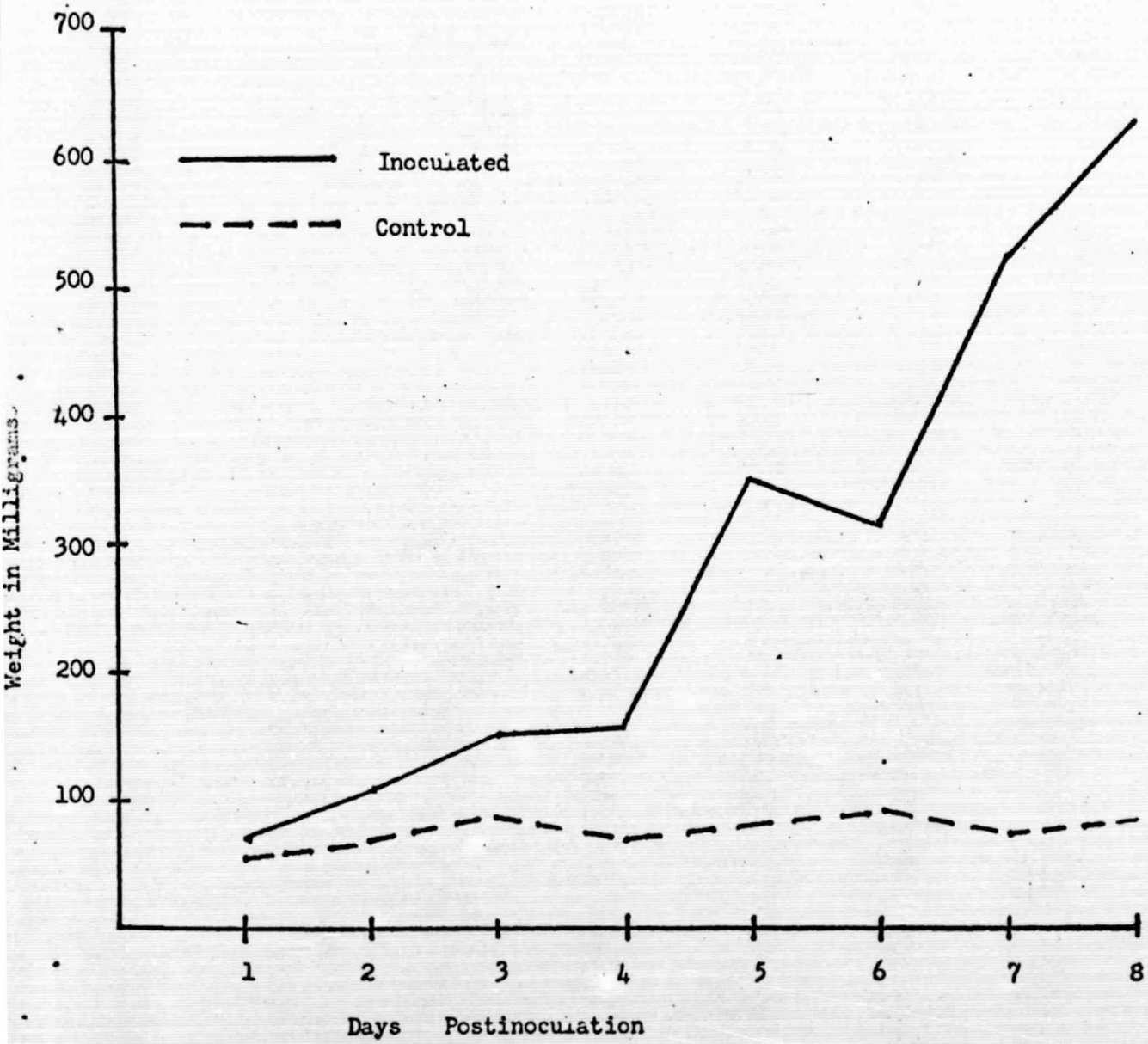


Figure 14. Plasmodium berghei in Hamsters

LDH Levels

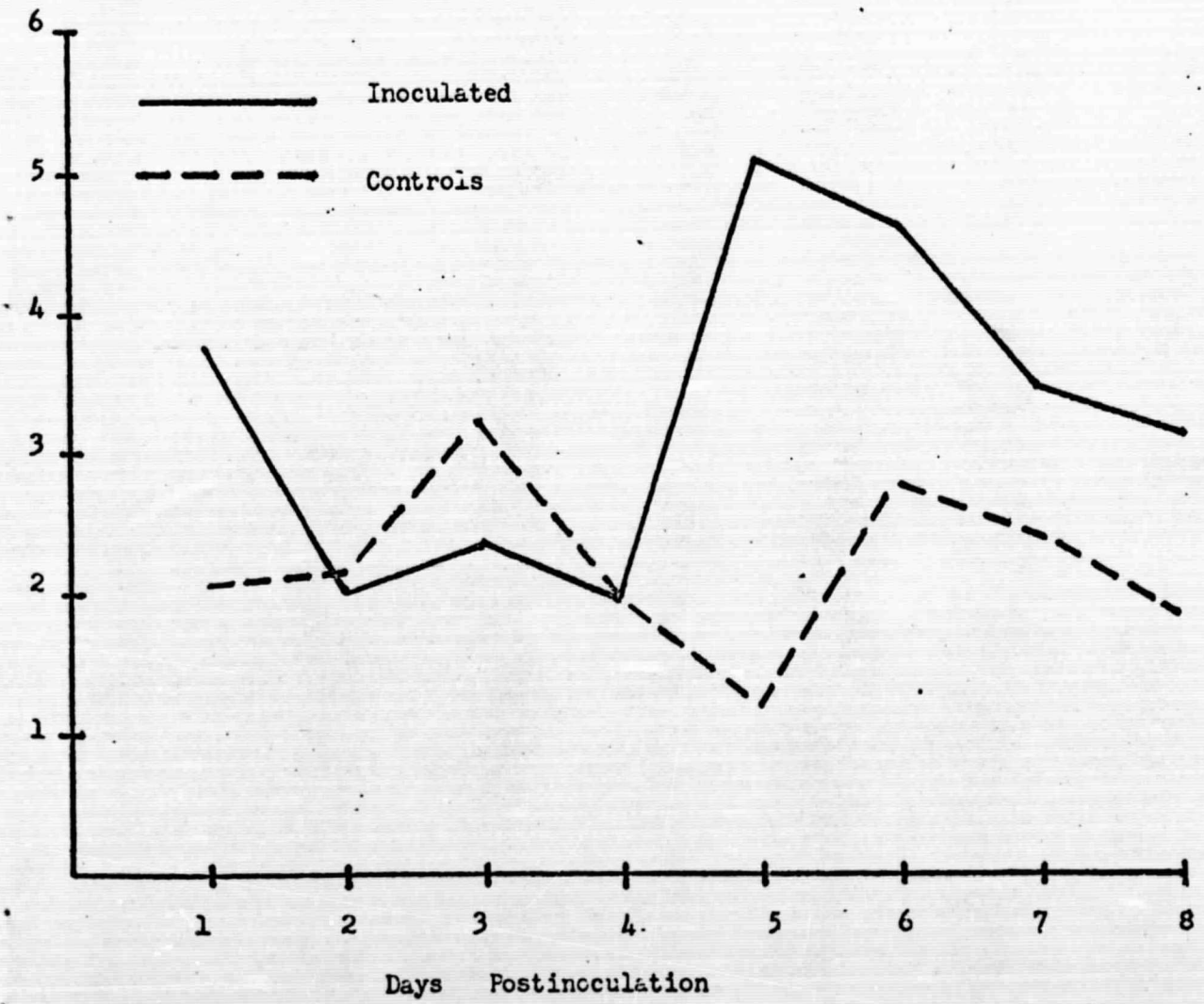


Figure 15. Plasmodium berghei in Hamsters

Parasitemia

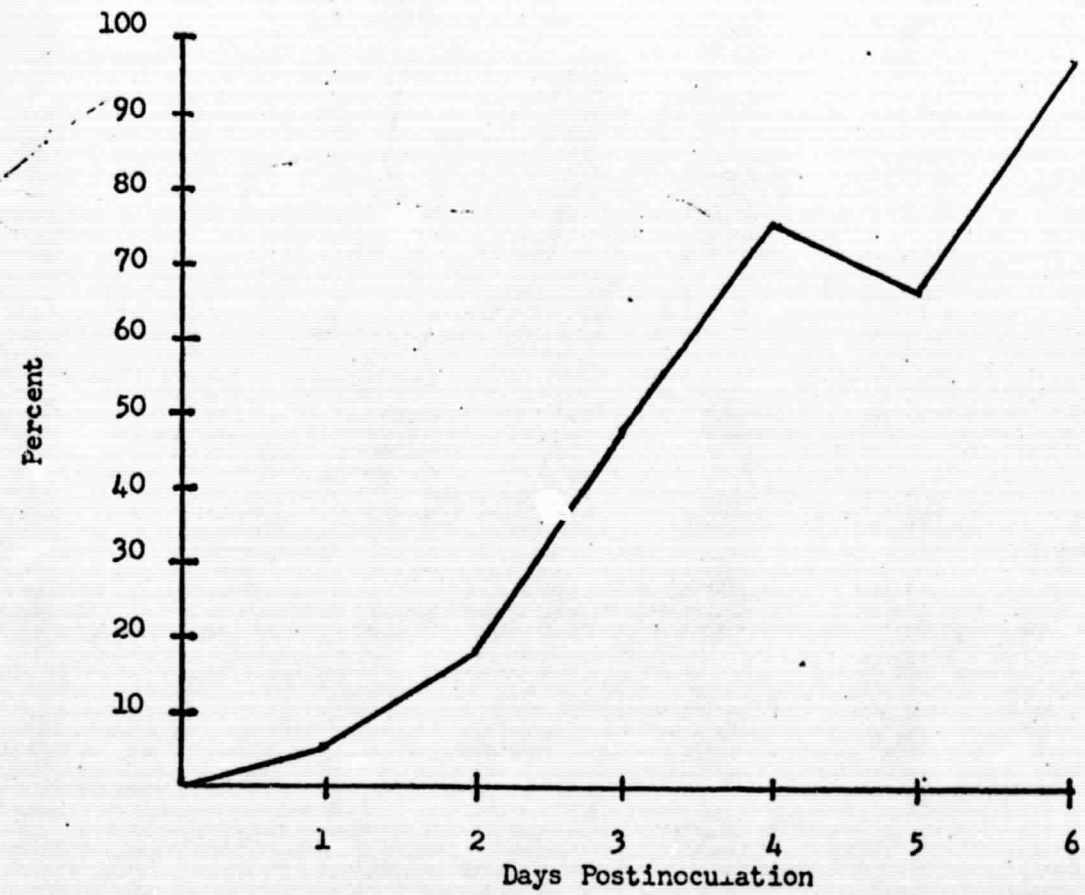


Figure 16. Plasmodium berghei in Hamsters

Hematocrit Values

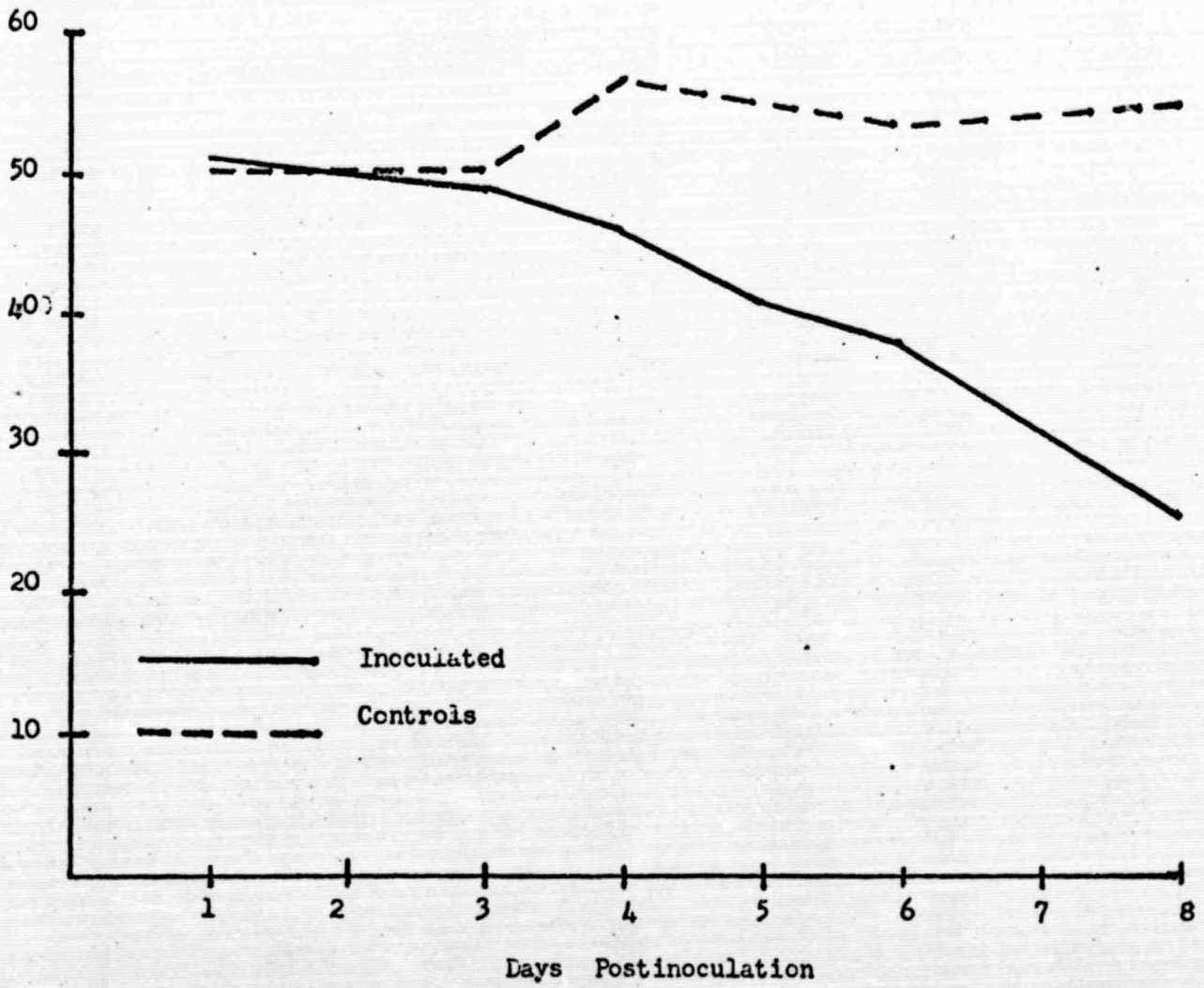


Figure 17. Titanium Dioxide Aerosolization

Top: Lung section taken after 24 hours exposure to TiO_2 aerosol. Note the macrophage containing dust particles.

Middle: Liver section containing TiO_2 Kupffer cells.

Bottom: A splenic follicle showing migration of TiO_2 from the marginal zone toward the peripheral zone of germinal center formation.

TABLE 1

Germinal Center (GC) Response to MHV

Experiment Number		Day	Number of Mice	GC Grade	Average Spleen Weight (mg.)
1.*	MHV	2	3	-	31
	Control**	2	2	-	25
2.	MHV	2	2	-	39
	Control**	2	2	-	42
1.	MHV	4	3	+++	42
	Control	4	2	+	30
2.	MHV	4	2	+++	30
	Control	4	2	++	49
1.	MHV	8	3	++++	50*
	Control	8	2	++	38
2.	MHV	8	2	++++	126
	Control	8	2	++	38
1.	MHV	16	3	++++	45
	Control	16	2	+++	33
2.	MHV	16	2	++++	71
	Control	16	2	++	65
1.	MHV	32	3	++++	68
	Control	32	2	++++	72
2.	MHV	32	2	++++	109
	Control	32	2	++++	39

*Experiment No. 1 represented the first test MHV_3 from first passage out of BALB/c tissue extract. In subsequent experiments, where the infectivity was markedly increased, the increase in splenic weight at day 8 was apparent.

**These controls represented mice inoculated with liver homogenates of normal mice. In addition to this control, uninoculated controls were maintained and examined as above.