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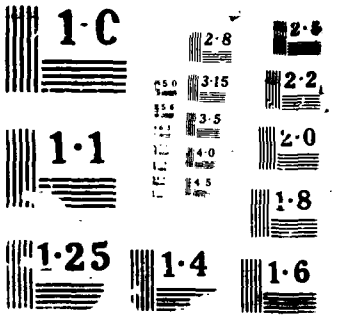
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Intraerythrocytic Killing of Malaria Parasites

Annual Report

Hannah Lustig Shear, Ph. D.

30 September 1987

Supported by

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New York University Medical Center

550 First Avenue

New York, New York 10016

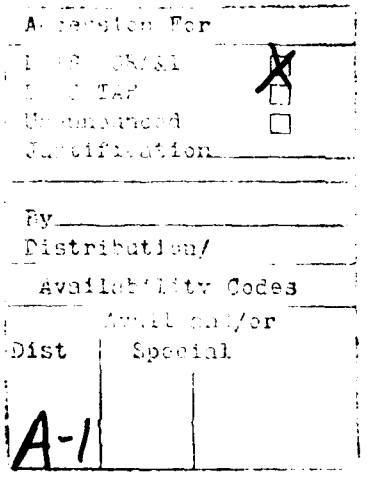
Summary

The purpose of these studies is to determine the role of activated macrophages in immunity to the blood stages of malaria. This is being accomplished by comparing the activity of macrophages during lethal and non-lethal malaria infections and in malaria-resistant and non-resistant mice. The results indicate that in non-lethal P. yoelii infection of mice, macrophage activity, as determined by H_2O_2 release, gamma-interferon production and lymphoproliferation, is high early in infection, declines during the time of peak parasitemia and recovers as the infection is cleared. Although some macrophage functions are enhanced during the early phase of lethal P. yoelli infection, this enhancement is not sustained. Recombinant gamma-interferon, infected into mice, had a protective effect in susceptible mice with lethal infections but not in non-lethal infections.

Foreword

Citations of commercial organizations and trade names in the report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)".



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ABSTRACT (Continue on reverse if necessary and identify by block number)

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Scientific Report

Research Problem

Evidence from this laboratory indicates that lymphokines (LK), which are produced by the spleen during rodent malaria infections, stimulate normal, resident macrophages to bind and ingest and to kill intraerythrocytic malaria parasites *in vitro* (1, 2).

Since the killing of the intraerythrocytic parasites *in vitro* appeared to be mediated, at least in part, by H_2O_2 secreted by macrophages (2, 3), studies were undertaken to determine whether levels of H_2O_2 production varied between lethal and non-lethal infections and between susceptible and resistant mice.

Further, since human gamma-interferon (Genentech, Inc.) was able to activate human monocyte-derived macrophages to kill *P. falciparum* (3) and preliminary evidence suggested that LK obtained from malaria-infected mice also contains gamma-interferon, studies were undertaken to determine the levels of gamma-interferon produced during lethal and non-lethal infections and in susceptible and resistant mice. In addition, the effect of recombinant mouse gamma-interferon (Genentech, Inc.) on the course of lethal and non-lethal rodent malaria was studied.

Background

Much evidence is accumulating to indicate that cell-mediated immune responses are very important in the response to some species of malaria (4) and may act as an adjuvant in vaccinated mice (5). However, the actual mechanisms whereby cell-mediated responses protect against malaria are not known.

The overwhelming evidence supports the concept that activated macrophages are involved in controlling several infections such as *Leishmania tropica* (6), *Rickettsia akari* (7), and *Trypanosoma cruzi* (8); that they are cytotoxic to tumor cells (9), and finally that they may be involved in vaccine-induced immunity (5).

We have been studying macrophage activation during rodent malaria (10) and the effects of macrophages activated with other stimulants on malaria-infected erythrocytes (1). Briefly, we have found that spleen cells of mice infected with BCG or malaria, produced factors or lymphokines (LK) which stimulated normal mouse peritoneal macrophages for enhanced phagocytosis of parasitized erythrocytes (1) and for killing of parasitized erythrocytes (2). We have also found that fresh monocytes or LK-stimulated, monocyte-derived macrophages were active in inhibiting the multiplication of *P. falciparum* (3). In both systems H_2O_2 seemed to be the active molecule. Killing was observed after parasitized erythrocytes bound to monocytes and was associated with an oxidative

burst in the monocytes. After the interaction, the parasitized erythrocytes appeared to be degenerating and looked like the previously described "crisis" forms (11).

Our results confirm and extend several other findings. Taliaferro and Cannon (11) observed that upon acquisition of immunity in monkeys infected with malaria, some intraerythrocytic parasites appear to degenerate within the erythrocytes. This finding implied that soluble mediators might affect malarial parasites. That such mediators might be secreted by macrophages was first suggested by Allison and Clark (12). Mice treated with BCG are protected against malaria and the mechanism suggested was that parasites are killed by products of activated macrophages. Since then, other parasiticidal factors have also been shown to have an effect on intraerythrocytic malaria parasites, namely, tumor necrosis factor (13), interferon (13) and a lipopolysaccharide-induced serum factor (14).

The concept that oxygen radicals might affect malaria parasites was suggested by the observations that injections of alloxan (15) and t-butyl hydroperoxide (16,17) into mice with P. vinckei (15, 17) or P. yoelii (16) markedly reduces parasitemia. These compounds generate reactive oxygen intermediates and their activity can be inhibited by iron-chelating agents such as desferrioxamine and diethylthiocarbamate. It is well known that malaria parasites are sensitive to oxidant stress. Cultures of P. falciparum grown in G-6-PD deficient erythrocytes are inhibited under high oxygen tension (18). In addition, dilutions of H_2O_2 as low as 10^{-5}M are toxic to P. yoelii and P. berghei in vitro and in vivo (19).

Early studies of Langhorne et al. (20) indicated that incubation of spleen cells from infected monkeys with parasitized erythrocytes reduced their ability to multiply. Later, Taverne et al. (21) demonstrated the killing of P. yoelii by cells of the monocyte-macrophage series. Data from this study also suggested that fresh blood monocytes or peritoneal cells activated by incubation with lymph node cells of immunized mice were more effective than normal peritoneal cells. Our studies show that H_2O_2 produced upon an oxidative burst in activated macrophages, is lethally damaging to P. yoelii and P. falciparum. Because these studies suggest an important protective mechanism in malaria, studies were undertaken to determine the roles of LK and H_2O_2 production in lethal and non-lethal P. yoelii infections and in susceptible and resistant mice.

Approach

We have taken the following approach in these studies:

1. In lethal and non-lethal P. yoelii infections in susceptible Balb/C ByJ mice we have determined:

a) the levels of H_2O_2 produced by peritoneal and splenic macrophages,

b) the levels of gamma-interferon produced by spleen cell cultures,

c) the ability of spleen cell cultures to proliferate in response to a mitogen (Con A) and to P. yoelii erythrocyte antigens.

2. Similar studies as in 1. above were carried out in more resistant CBA/J mice.

a) H_2O_2 production
b) gamma-interferon production
c) responses to Con A and P. yoelii antigen.

3. We have determined the effect of mouse gamma-interferon on the course of lethal and non-lethal P. yoelii infections in outbred mice and inbred susceptible (Balb/C ByJ) and resistant (CBA/J) mice.

Results

1. a) The levels of H_2O_2 produced by peritoneal and spleen macrophages measured as in (21), over the course of either P. yoelii17xL (lethal) or P. yoelii17xNL (non-lethal) in Balb/C ByJ mice is shown in Fig. 1. In the non-lethal infection, H_2O_2 levels are increased above normal by day 5, decline during the second week of infection (when parasitemia peaks) and then begin to increase after week two. H_2O_2 production by peritoneal macrophages from mice infected with P. yoelii17xL were not above control levels and declined to below control levels until the mice died. Spleen cells did have increased H_2O_2 production early in the lethal infection, however, this declined and did not recover.

b) Gamma-interferon levels produced by spleen cell cultures pulsed with mitogen or P. yoelii antigen were measured by RIA as in (22). In the non-lethal infection, levels of gamma-interferon were greatly increased 4-5 days after infection, declined during the second week and then began to recover to control levels as the infection was cleared (Fig. 2). In the lethal infection, however, gamma-interferon production was at about control levels (gamma-interferon produced by spleen cells from uninfected mice pulsed in the same way) throughout the infection.

c) The proliferative response of spleen cells to the T cell mitogen, Con A, and to a preparation of erythrocytic stage P. yoelii antigens (prepared as in 2) followed the same general pattern as the production of H_2O_2 and gamma-interferon (Fig. 3). The response to both Con A and P. yoelii was high on day 3, dropped to below control levels by day 12 and then recovered in the non-lethal infection. In the lethal infection the responses were high on day 3, dropped to below control levels and then the animals died.

2. The same three assays were performed on peritoneal and spleen cells from the more resistant strain of mice, CBA/J. These mice generally survive infection with 10^4 parasitized erythrocytes of P. yoelii17xL. The results are shown in Figs. 4-6. H_2O_2 release from peritoneal and spleen cells was well above control levels on day 3 of infection, declined to control (peritoneal cells) or below control (spleen cells) levels from days 5-20, and then recovered. During the lethal infection the H_2O_2 response was initially high, decreased dramatically on day 5 (peritoneal) or day 10 (spleen) and then recovered temporarily. The gamma-interferon response of cells triggered with Con A or antigen was also high initially, declined during the second week of infection and recovered in both non-lethal and lethal infections. Interestingly, the proliferative responses to P. yoelii antigen was high initially, declined and recovered temporarily in the non-lethal infection. However, in the lethal infection there was a different pattern, the response was low initially, peaked during the second week and then declined to below control levels.

3. Effect of gamma-interferon on P. yoelii infections in vivo.

SW female mice, 4-6 weeks old were injected with doses of gamma-interferon ranging from 1,000 to 100,000 units/day, intraperitoneally. Controls received either diluent, gamma-interferon plus anti-gamma-interferon or thioglycollate. Fig. 7 shows that mice infected with P. yoelii17xL were protected by gamma-interferon in a dose-dependent fashion. Controls were not protected. However, mice infected with the non-lethal P. yoelii did not show the same degree of protection (Fig. 8).

Further experiments were undertaken in inbred mice. In susceptible, Balb/C ByJ, gamma-interferon had a protective effect against P. yoelii17xL but no effect on the non-lethal infection (Fig. 9, 10). Interestingly, in the more resistant CBA/J mice there was only a slight protective effect against P. yoelii17xL (which is not lethal in these mice) and no effect on the non-lethal parasite (Fig. 11, 12).

Discussion and Conclusions.

Our initial observations, that there are indeed differences in the macrophage response to lethal and non-lethal P. yoelii infections made during the first year of this project, have now been confirmed and extended with more information on the time course of these changes and the differences between susceptible and resistant strains of mice. In addition, the responses of peritoneal cells compared with spleen cells have been analyzed.

The data indicates that the mice make an early macrophage response to both lethal and non-lethal P. yoelii as indicated by H_2O_2 production by both peritoneal and spleen cells. In the non-lethal infection, the decline in H_2O_2 production is followed

by a recovery whereas in the lethal infection, it is not. These results are in parallel with the cellular response to infection. Both production of gamma-interferon and lymphoproliferation in response to P. yoelii antigen and a T cell mitogen, Con A, show a similar pattern. In non-lethal infections there is a good early response of gamma-interferon, a decline, and a slight recovery. In lethal infections there is hardly any gamma-interferon response at all. Even more striking is the lymphoproliferative response in which spleen cells from mice infected with non-lethal malaria show a striking recovery as the infection is cleared while mice infected with lethal malaria do not.

In more resistant mice (CBA/J), P. yoelii17x is not lethal. Thus, the responses to both lethal and non-lethal P. yoelii more closely resemble the response of Balb/C mice to P. yoelii17xNL, i.e., a good early response, a decline as parasitemia increases and then a recovery. The only exception to this pattern was the lymphoproliferative response of mice infected with P. yoelii17xL. The reason for this is under investigation.

The results of injection of exogenous interferon are interesting in view of the results discussed above. Gamma-interferon appeared to have an effect primarily in the lethal P. yoelii infection. Thus, in animals with poor macrophage responses, gamma-interferon helps. If the animal is making a sufficient response, gamma-interferon has no effect.

Recommendations

During the coming year it will be important to determine how gamma-interferon is mediating its effect on the erythrocytic cycle of malaria. Preliminary experiments indicate that macrophages from mice injected with gamma-interferon show high levels of H_2O_2 production and enhanced phagocytosis. However, controls injected with gamma-interferon plus a monoclonal antibody against gamma-interferon also show the same effect. Since this might be due to the injection of immune complexes, studies are being planned in which $(Fab)_2$ fragments of the monoclonal will be injected with gamma-interferon and a polyclonal antiserum against gamma-interferon will be raised in rabbits and $(Fab)_2$ fragments of this antiserum will be utilized. Further, these reagents will be useful in experiments to see the effect of anti-gamma interferon on lethal and non-lethal P. yoelii infection. These experiments are planned for the coming year.

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Fig 8. H_2L_2 Response in DHLB/LCBYJ Mice

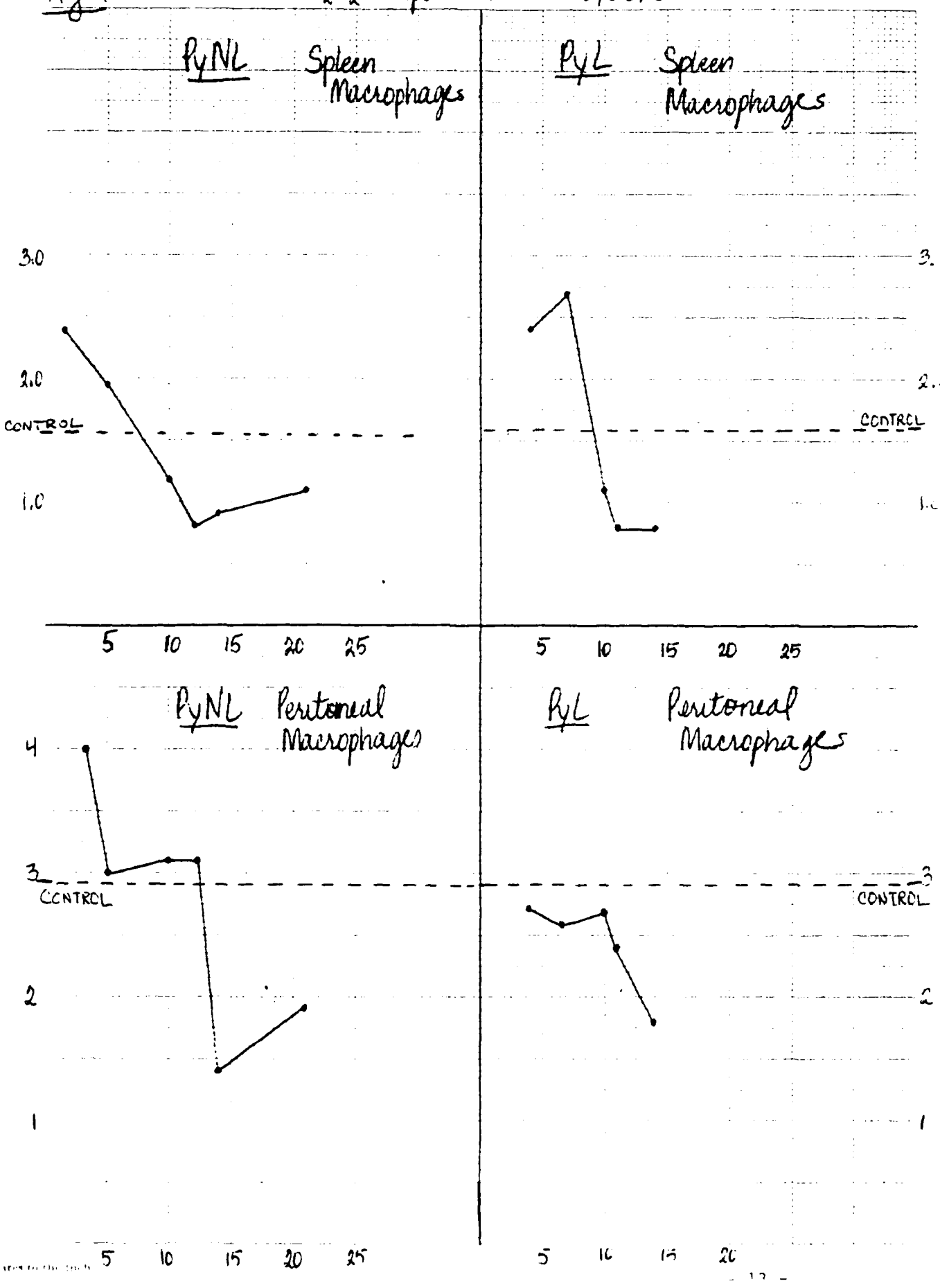


Fig 2. 0-LFN Response in DMSO/CBYJ mice

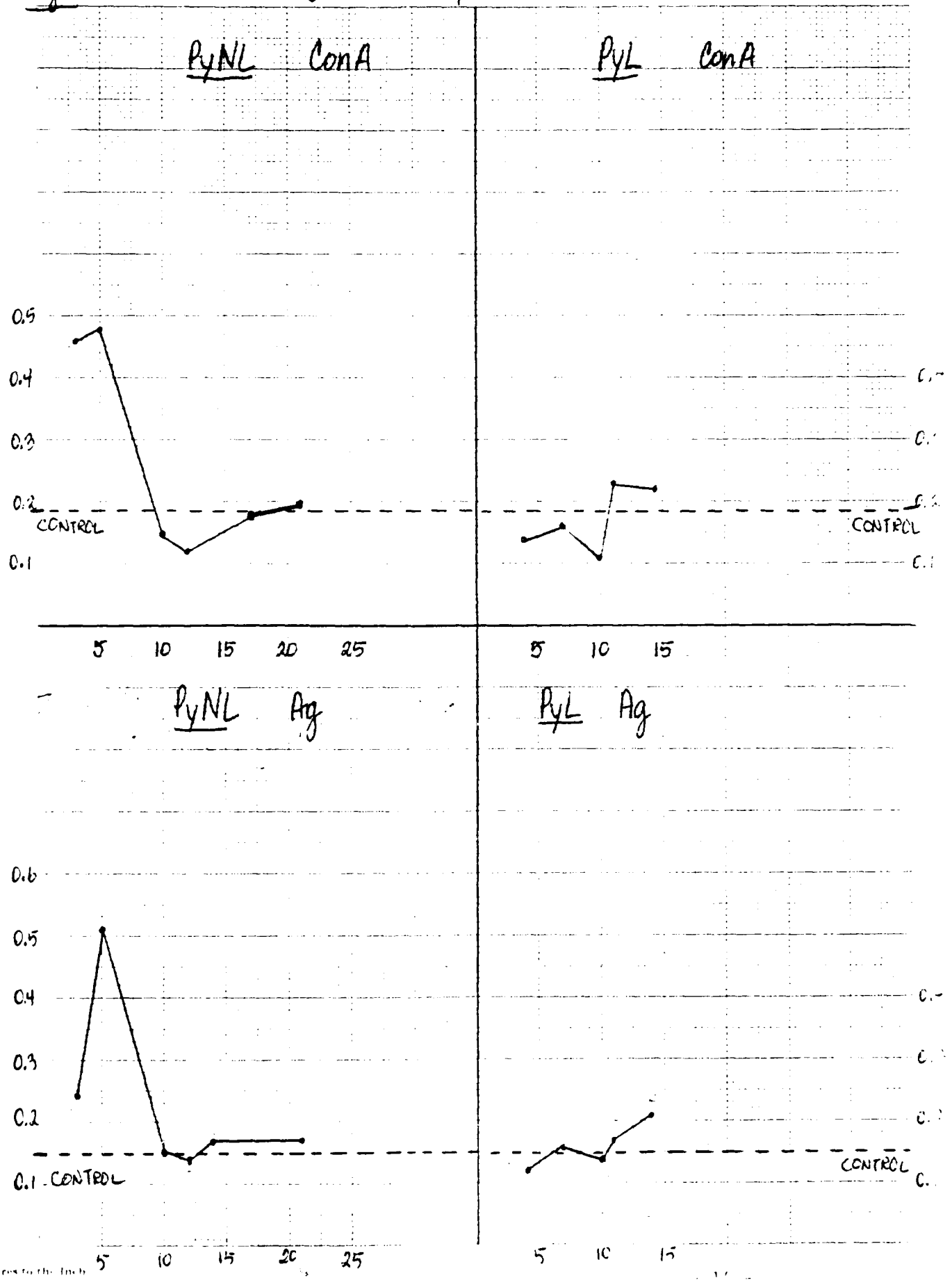


Fig 3. Lymphoproliferative Response in BALB/CBYJ

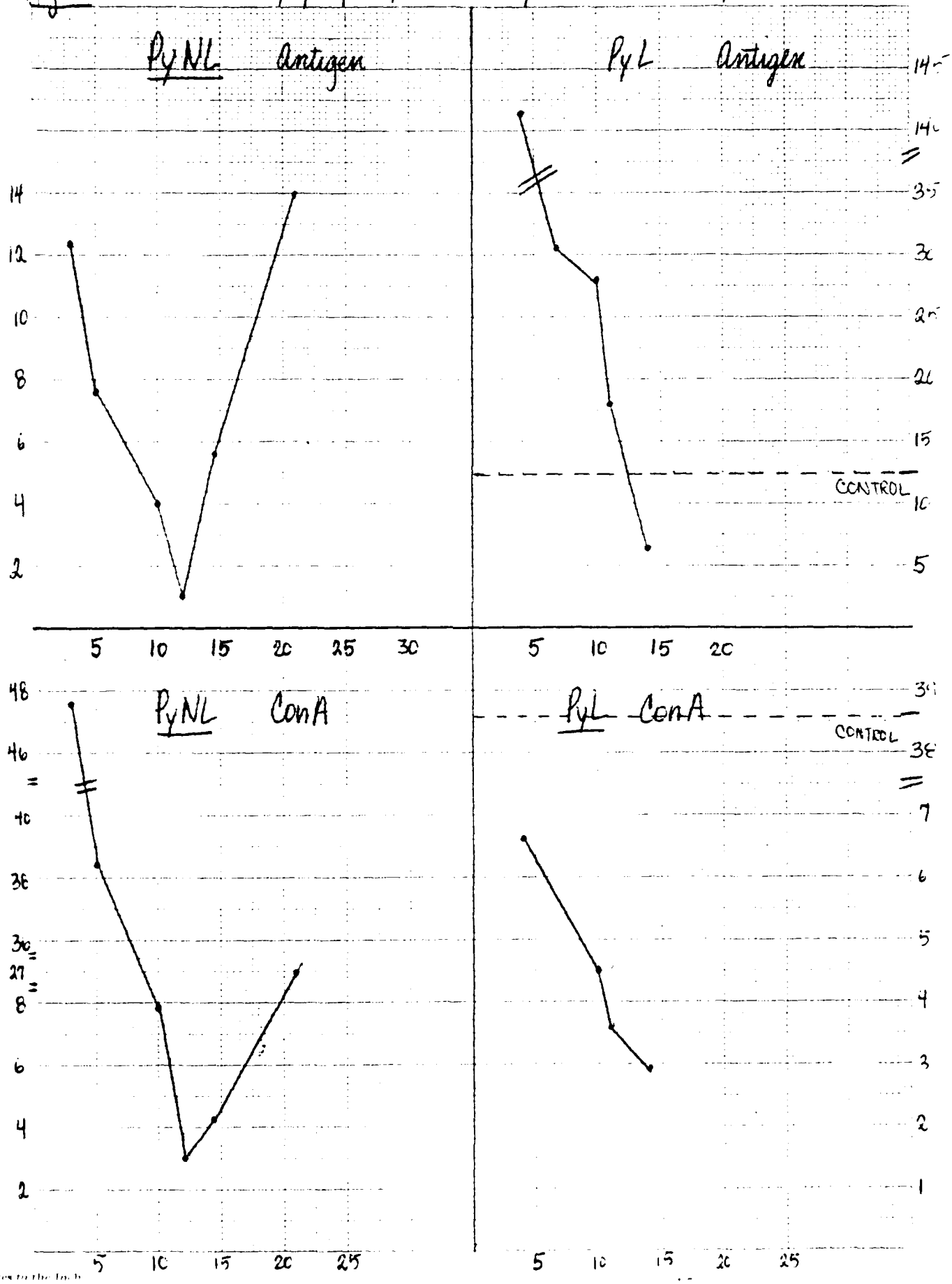
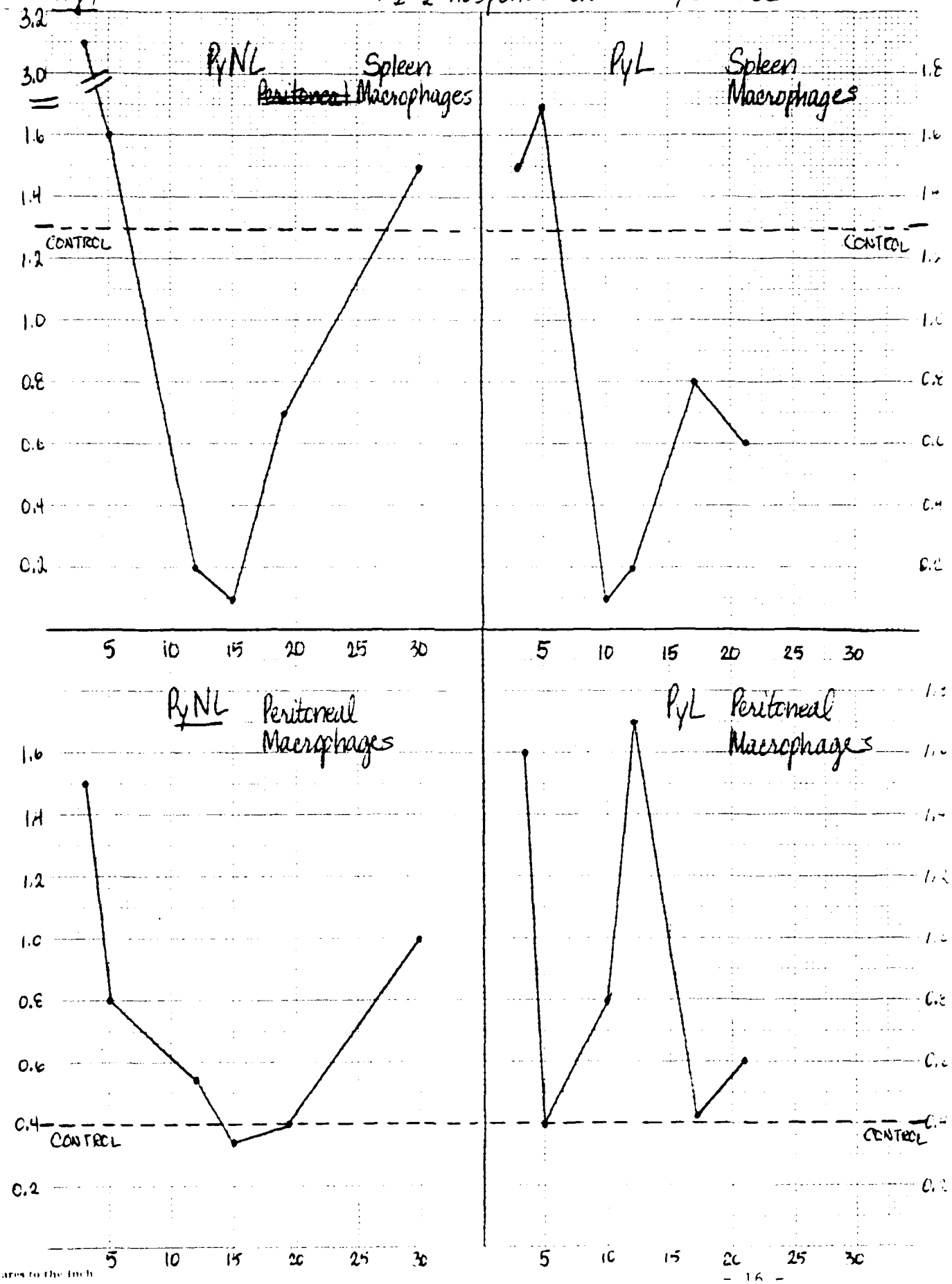


Fig 4 H_2O_2 response in COH/J mice



Figs. Con A

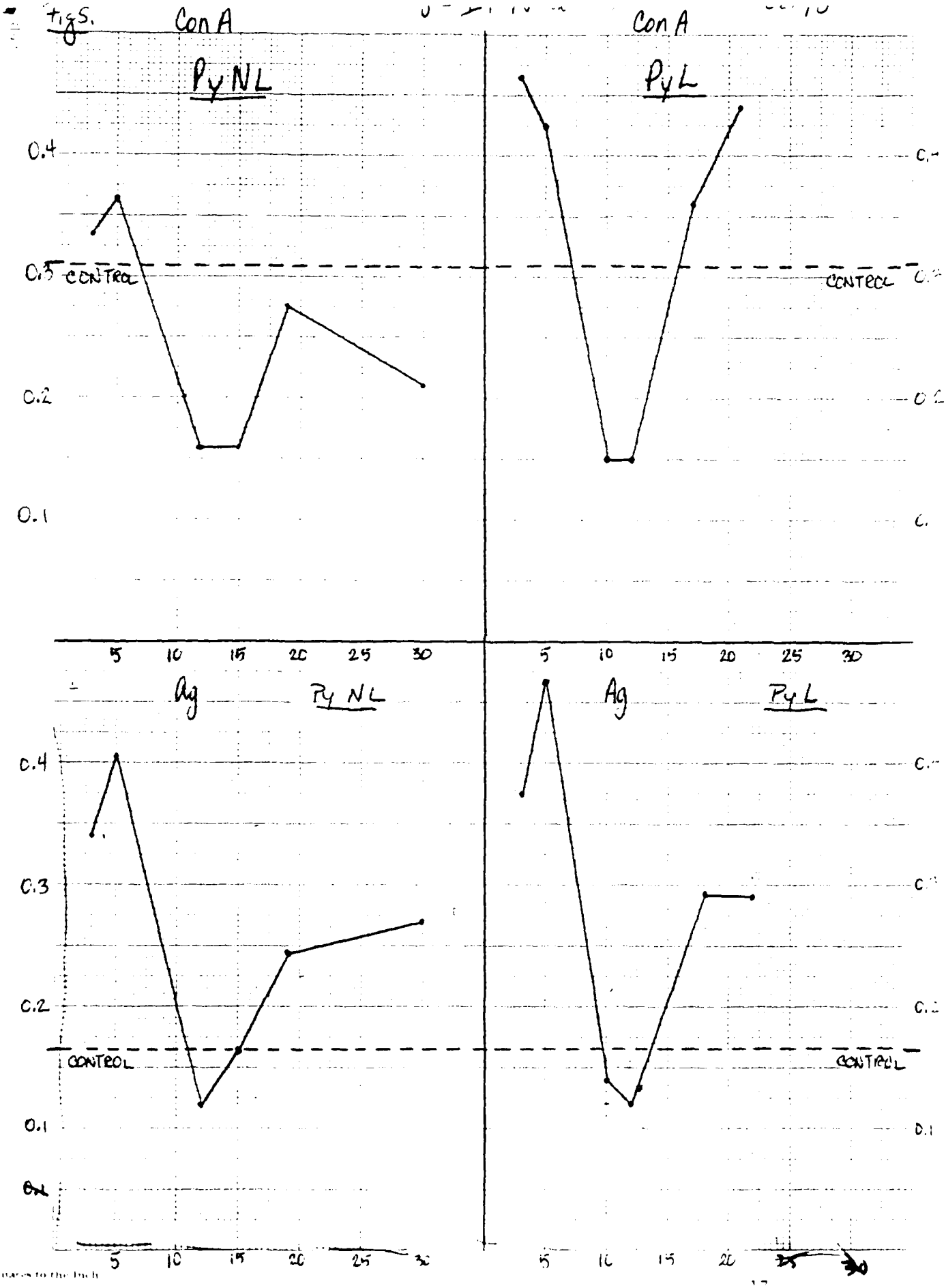
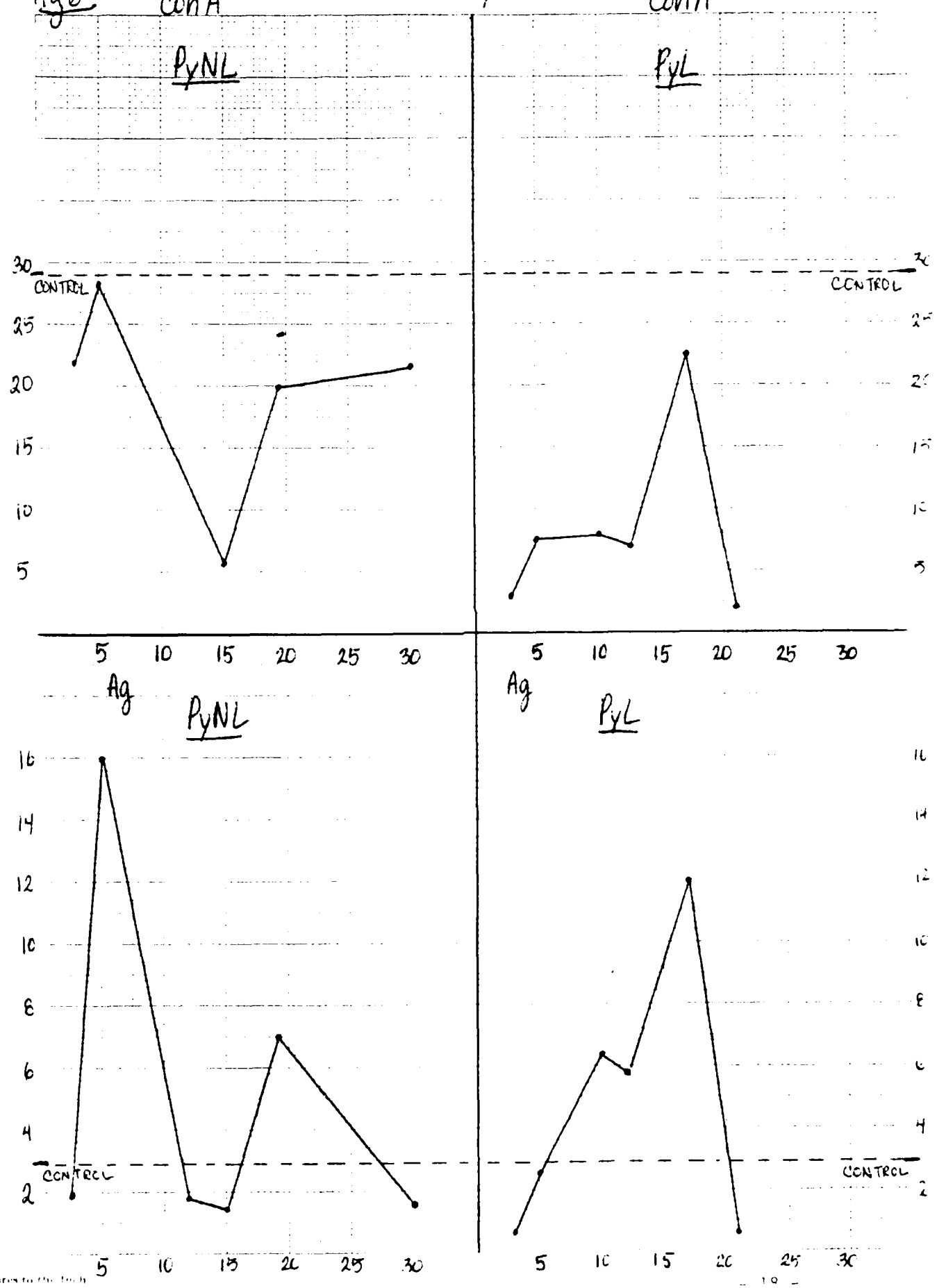


Fig 6


Con A
PyNL
Con A
PyL
30
CONTROL
25
20
15
10
5
5 10 15 20 25 30
Ag
PyNL
Ag
PyL
16
14
12
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8
6
4
CONTROL
2
5 10 15 20 25 30

Fig 7.

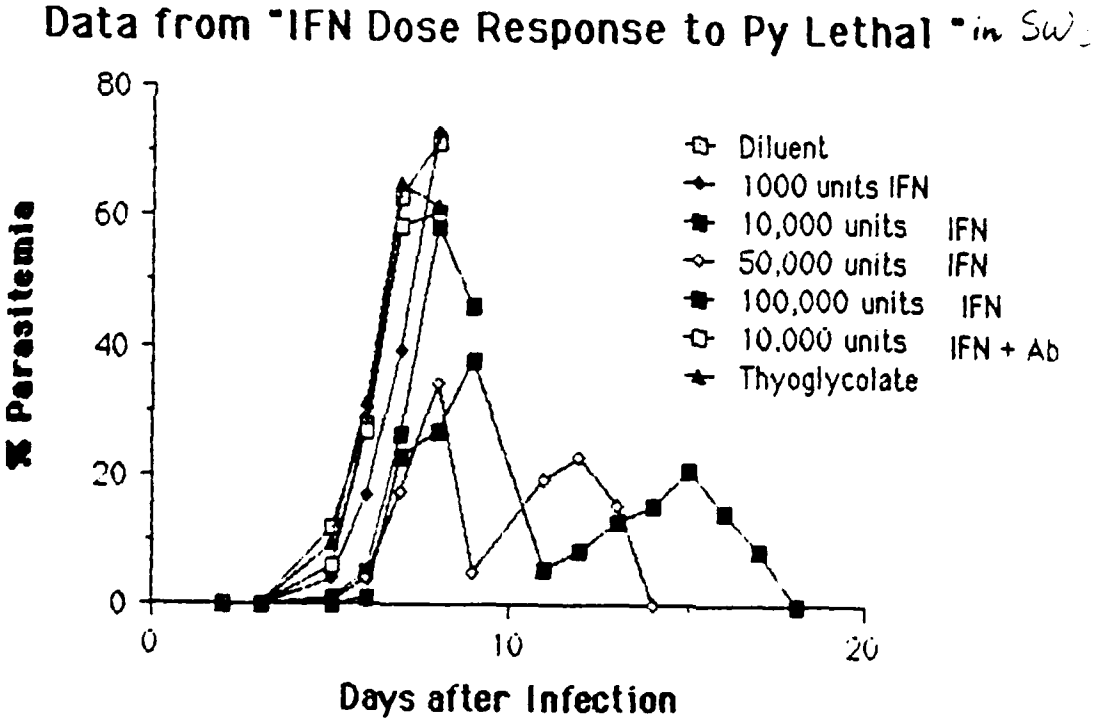
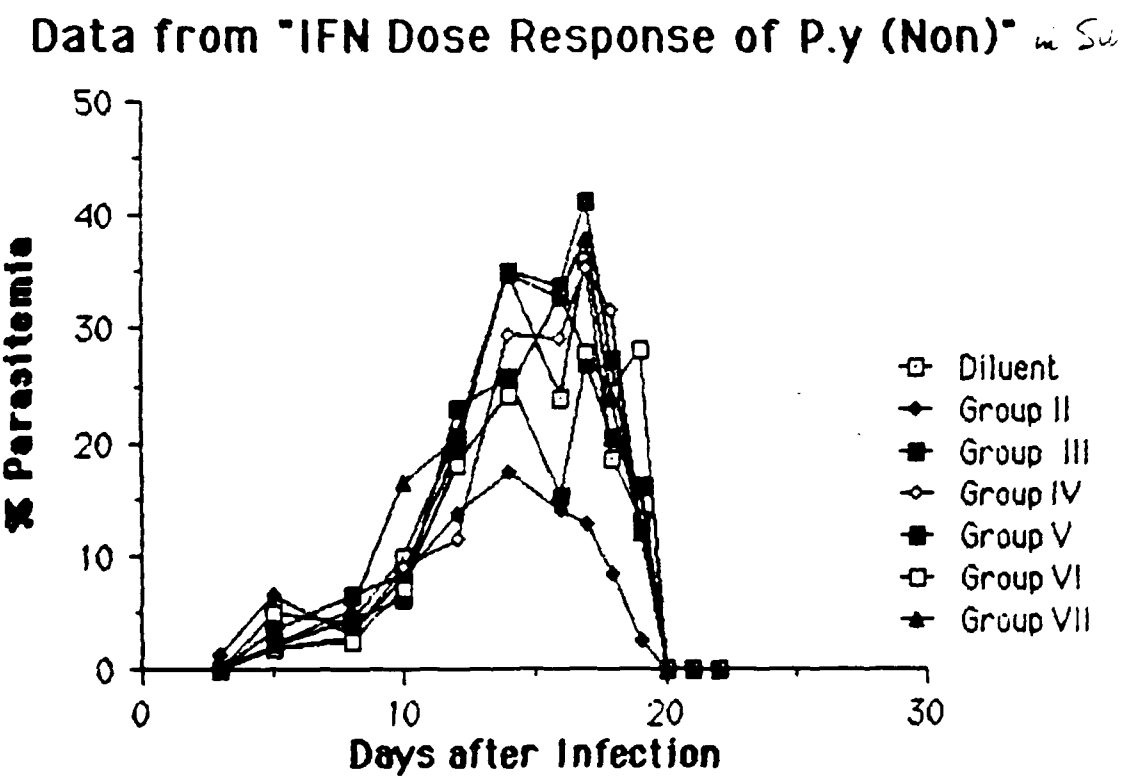

Data from "IFN Dose Response to Py Lethal "in Sw
% Parasitemia
80
60
40
20
0
0
10
20
Days after Infection
Diluent
1000 units IFN
10,000 units IFN
50,000 units IFN
100,000 units IFN
10,000 units IFN + Ab
Thyoglycolate

Fig. 8


Data from "IFN Dose Response of P.y (Non)" in Su
% Parasitemia
50
40
30
20
10
0
0
10
20
30
Days after Infection
Diluent
Group II
Group III
Group IV
Group V
Group VI
Group VII

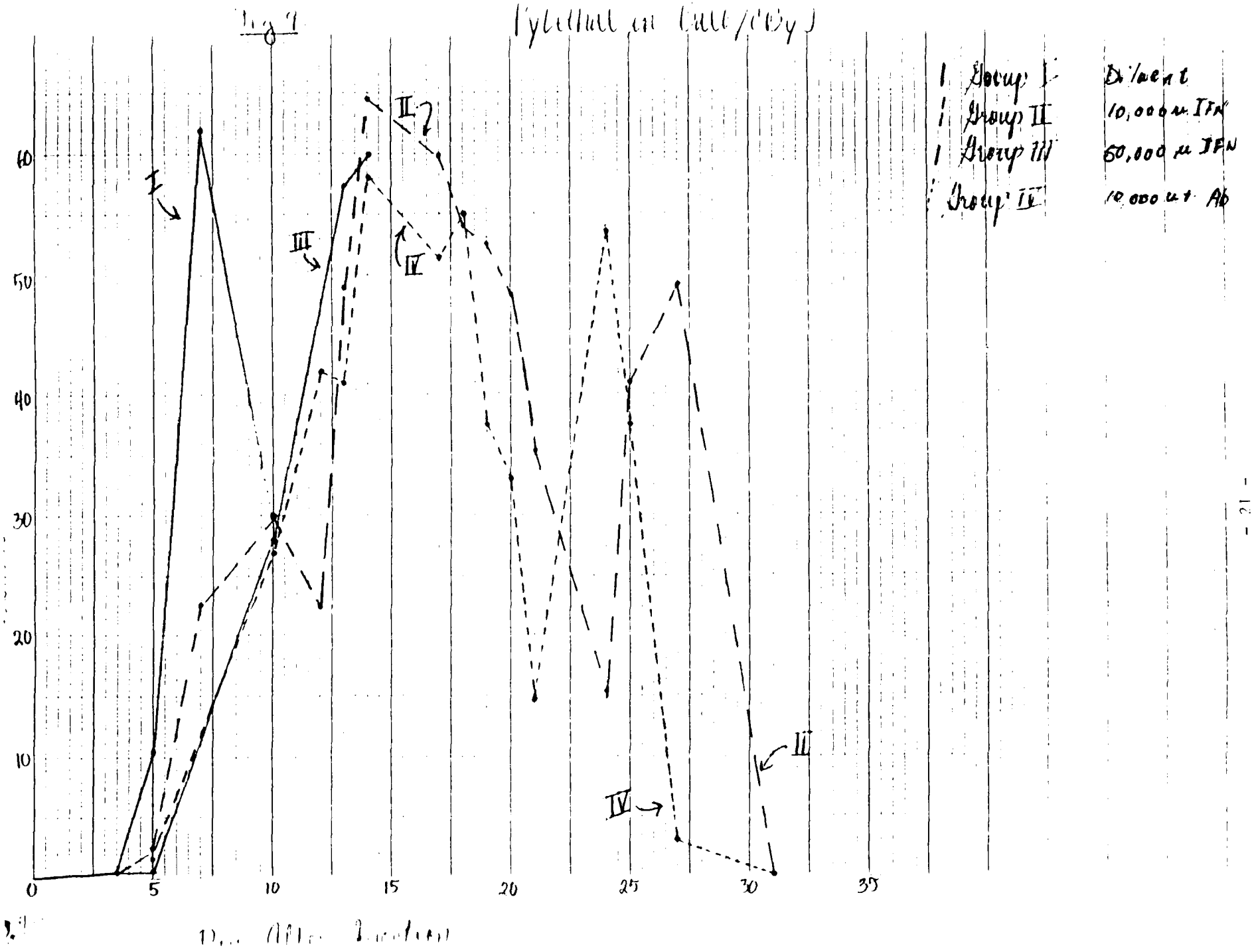
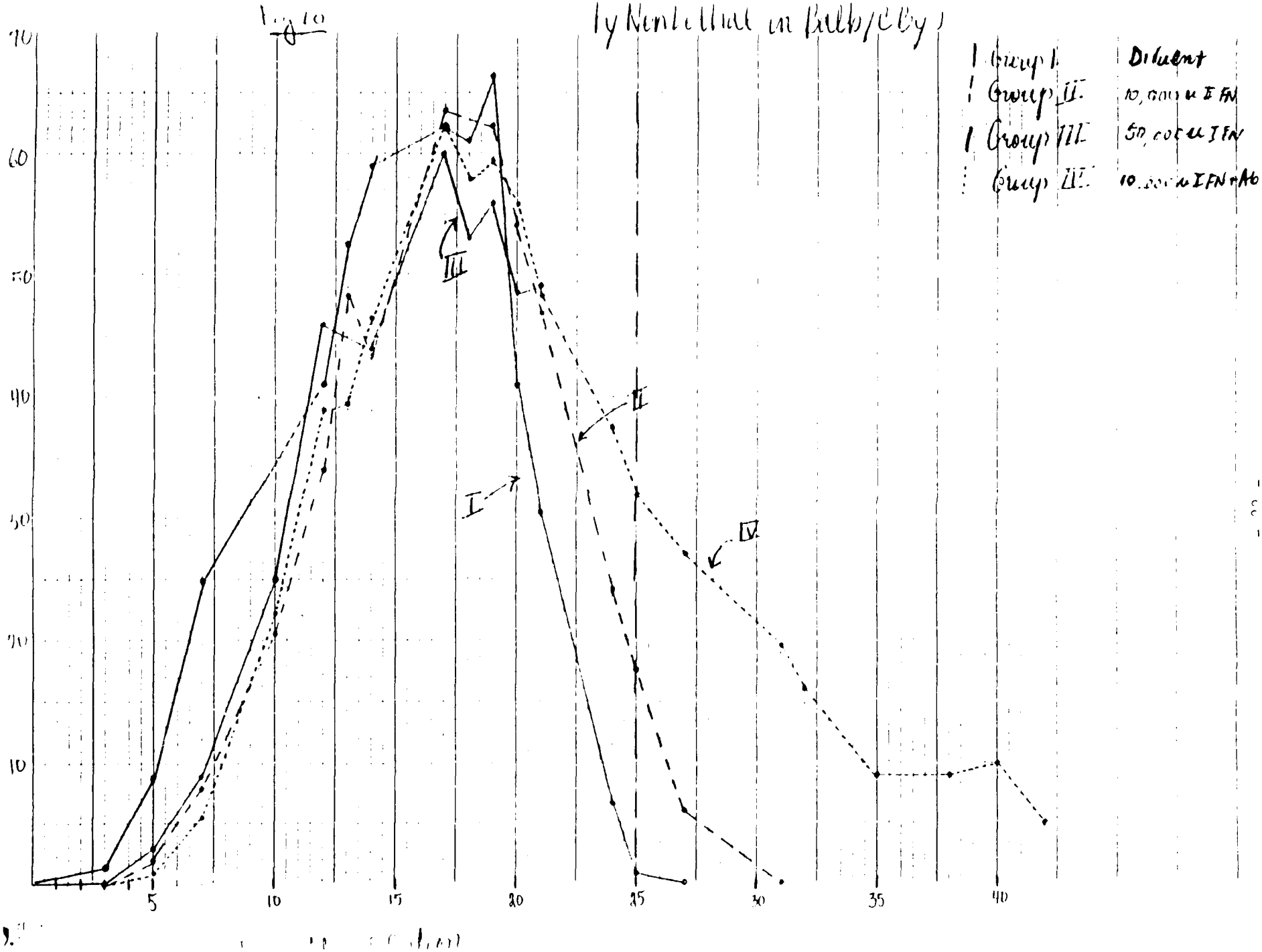


Fig 1
Cytotoxicity (in Cell/CBy)
Group I - Diluent
Group II 10,000 u IFN
Group III 50,000 u IFN
Group IV 10,000 u IFN Ab
I
II
III
IV
0
5
10
15
20
25
30
35
10
20
30
40
50
60


Log 10
ly Neutralized in Bulb/CBy)
Group I
Group II
Group III
Group IV
Diluent
10,000 u IFN
50,000 u IFN
10,000 u IFN + Ab
90
60
50
40
30
20
10
5
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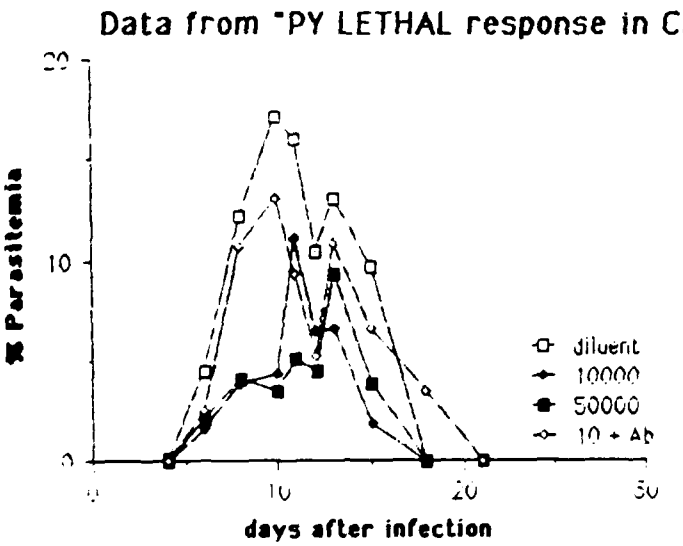
40
I
II
III
IV

Fig. 11


Data from "PY LETHAL response in CBA/J"
% Parasitemia
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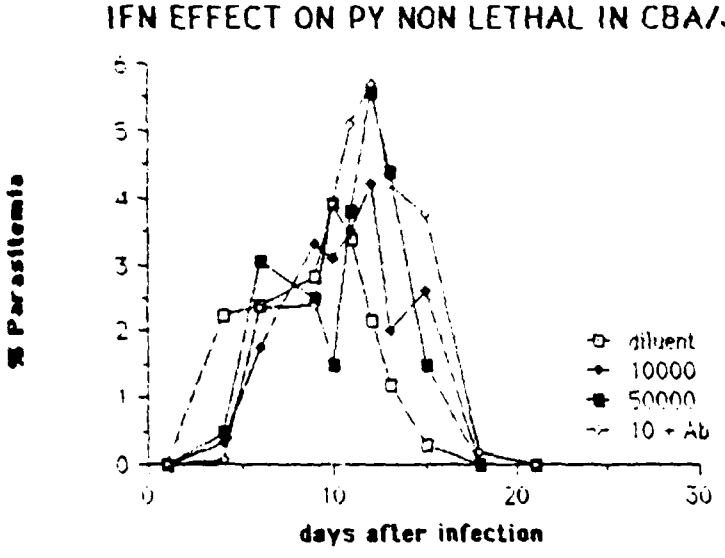
10
0
0
10
20
30
days after infection
diluent
10000
50000
10 + Ab

Fig 12


IFN EFFECT ON PY NON LETHAL IN CBA/J
% Parasitemia
0
1
2
3
4
5
6
0
10
20
30
days after infection
diluent
10000
50000
10 + Ab

Appendix

Legends to Figures

Fig. 1. H_2O_2 response of spleen and peritoneal marophages in Balb/C ByJ mice infected with P. yoelii17xL or P. yoelii17xNL. Mice were infected I.P. with 10^8 parasitized erythrocytes. On the days indicated, animals were sacrificed, peritoneal and spleen cells harvested, plated and H_2O_2 measured. Data represents the mean of two mice/point.

Fig. 2. Gamma-interferon response of spleen cells from Balb/C ByJ mice infected with P. yoelii17xL or P. yoelii17xNL. Mice were infected as for Fig.1. On the days indicated, animals were sacrificed, spleen cells obtained and cultured for 3 days at 5×10^5 cells/ml. On day 3, 50 ul of the supernatant was assayed for gamma-interferon by RIA. Data represents the mean of triplicate assays from 2 mice/point.

Fig. 3. Lymphoproliferative response of spleen cells in Balb/C ByJ mice infected with P. yoelii17xL or P. yoelii17xNL. Animals were infected as in Fig. 1. On the days indicated, mice were sacrificed, spleen cells obtained and cultured at 5×10^5 cells/ml for 4 days. On the 3rd day, cells were pulsed with thymidine and harvested on day 4. Data represents the mean of triplicate cultures from 2 animals/point.

Fig. 4. H_2O_2 response of spleen and peritoneal macrophages in CBA/J mice infected wuth P. yoelii17xL or P. yoelii17xNL. As for Fig. 1.

Fig. 5. Gamma-Interferon response of spleen cells from CBA/J mice infected with P. yoelii17xL or P. yoelii17xNL. As for Fig. 2.

Fig. 6. Lymphoproliferative response of spleen cells from CBA/J mice infected with P. yoelii17xL or P. yoelii17xNL.. As for Fig. 3.

Fig. 7. Effects of recombinant gamma-interferon on the course of P. yoelii17xL in SW mice. Mice were pretreated I.P. for 3 days with the materials shown, in 0.2 ml. On day 0 they were infected with 10^4 infected erythrocytes I.P. Injections were continued daily. Data represents the mean of 5 mice/group.

Fig. 8. Effect of recombinant gamma-interferon on the course of P. yoelli17xNL in SW mice. As for Fig. 7.

Fig. 9. Effect of recombinant gamma-interferon on the course of P. yoelii17xL in Balb/C ByJ mice. Mice were pretreated I.P. for 3 days with the materials shown, in 0.2 ml. On day 0 they were infected with 10^4 parasitized erythrocytes I.P. Injections were continued daily to day 28 except for days 8, 9, 15, 16, 22, 23. Data represents the mean of 4 mice/group.

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