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Immunomodulation in vivo during Tularemia in AKR/J Mice

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Running Head: IMMUNOMODULATION DURING TULAREMIA

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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ABSTRACT

The anti-sheep red blood cell (SRBC) primary in vivo immune response was used to measure immunomodulation during tularemia infection in AKR/J male mice. Three periods of altered immune response ability were noted in the 30-day period after subcutaneous inoculation. These effects, in sequence, were: early augmentation, suppression with maximum at day 12 and late augmentation at day 30. These altered immune responses correlated most directly to Francisella tularensis antigen load in the spleen. However, other infection-related phenomena (notably decreased nutrition) occur which may cause some immunomodulation.

The major events leading from immunogenic stimulation to antibody production have been elucidated. Some of the control and regulatory processes determining the amount and extent of these events have been determined, while the entire area of regulation is under intense research scrutiny. Quality and quantity of antigen have been shown in numerous studies to be of critical importance in influencing antibody production. Immune response during bacterial infection, when judged by these criteria, presents an incredibly wide variety of opportunities for impact on regulatory functions. The amount and type of antigenic stimuli are affected by the bacterial proliferation and multiple immunogenic components. Moreover, phagocytosis and killing of bacteria impart a further dynamic state of antigens present. Many possibilities for altered interactions of cell subpopulations can be hypothesized due to any of these effects. A first approximation of in vivo effects may be helpful in dissecting and understanding these processes. The purpose of this work is to provide this sort of overview by observing primary immune induction to sheep red blood cells (SRBC) during the course of tularemia infection.

The infectious disease used as the model is the live, vaccine strain (LVS) of Francisella tularensis in AKR/J male mice. Within the spectrum of virulence exhibited by various strains of F. tularensis, LVS is a low virulence strain used to vaccinate humans at risk to more virulent strains. The disease process and production of immunity has been likened to that of Listeria monocytogenes (6). Immunity is much more dependent on cellular processes than specific antibody (6-9, 12, 17). On the basis of bacterial recovery, target organs are those organs with significant numbers of reticuloendothelial cells. LVS,

when administered to mice, produces an acute infection with variable severity depending on the factors noted above, plus the state of health and strain of the mice (3).

In this study, the kinetics of bacterial recovery and spleen and liver size variations were correlated to changes in ability to respond to the antigenically unrelated, T-dependent antigen, SRBC. Results show that both augmentative and suppressive episodes of immunomodulation occur during LVS infection. The changes are correlated with bacteria in the body and specifically in the spleen.

MATERIALS AND METHODS

Mice. Male AKR/J mice, 6-8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, Maine). Correlation of data to splenomegaly dictated that mice be examined carefully for wounds. Mice with wounds caused by chewing, etc., were discarded due to potentially enlarged spleens and variation in their immune response ability. Obviously enlarged spleens from normal appearing mice were not used.

Bacterial infection. A dilution of lyophilized F. tularensis vaccine (LVS), lot 11 (National Drug Co., Philadelphia, Pa.) was inoculated either subcutaneously (s.c.) at an inguinal site or intravenously (i.v.) in a tail vein in 0.2 ml gel saline. Subsequent growth of inoculum on glucose-cysteine-blood agar (GCBA) plates showed that the mice received doses ranging from $1-1.5 \times 10^3$ viable organisms.

In vivo SRBC immunization. SRBC (Colorado Serum Co. Laboratories, Denver, Col.) received in 50% Alsever's solution, were washed in physiological saline, centrifuged and resuspended to a concentration of 2.5×10^8 cells/ml. One milliliter of this suspension was inoculated intraperitoneally (i.p.) into mice at varying times after s.c. LVS inoculation. Assay for specific direct plaque-forming cells (DPFC) to SRBC was performed 5 days after SRBC inoculation in all cases.

Several factors affected the numbers of specific plaque-forming cells (PFC) in vivo from normal mice. Experience has shown that aged SRBC and numerous procedural factors are the greatest source of intergroup variations. These effects are greatly compounded by the broad range of reactivities found in intragroup normal mice. For these reasons control groups were assayed on each day that LVS-infected groups were tested.

Spleen cell preparation. Mice were killed by cervical dislocation and spleens were removed aseptically. Spleen cell suspensions were prepared in Hanks' balanced salt solution (HBSS) without bicarbonate. Suspensions were allowed to settle in tubes on ice for 5 min to remove tissue debris. The resulting suspensions of discrete cells were decanted, counted on a Coulter counter (Model Zf), and diluted to desired concentrations. Spleen were handled individually and cells from different spleens were not pooled. Cells were 90-95% viable prior to assay by trypan blue dye exclusion.

Plaque assay for direct anti-SRBC antibody-forming cells. The number of antibody-forming cells after in vivo SRBC immunization was determined by the microscope slide modification of the Jerne plaque assay (5, 15). All data are of direct PFC only.

Calculation and comparison of mean PFC for normal and infected mice. At least six mice were used per group. Two concentrations of each spleen cell preparation were regularly placed on triplicate slides for assay of PFC. One of these two was chosen for each mouse. Values were normalized to spleen size, where appropriate, and a mean was determined for each triplet. Mean $\pm$ S.E. was determined for PFC and also for number of spleen cells harvested for each group of six or more mice. The statistical two-sample problem format was used for comparison of controls and treated mice. Student's t test was applied to determine significance of the data.

Bacterial enumeration in spleen, liver and blood. Blood was drawn by intracardiac puncture; 0.2 ml was plated on glucose cysteine blood agar (GCBA) plates. Liver and spleen were excised aseptically from mice killed by cervical dislocation, minced with iris scissors and ground

with a manual glass homogenizer in 2 ml of gel saline. Each tissue was serially diluted in sterile gel saline and inoculated in triplicate on dry GCBA plates. Colony counts were made after 72-h incubation at 37°C.

RESULTS

Modulation of anti-SRBC primary in vivo immune response. Table 1 and Figures 1A and B present the results of numbers of DPFC. These data are a composite of seven experiments, with seven mice in each group. Table 1 shows the data used to plot the graph of Figure 1A, while the bar graph of 1B is a more easily visualized plot of the same data. The numbers of DPFC in infected mice were greater than normal on day 5, less than normal on day 12 and again greater than normal on day 15.

Change in size of liver and spleen in response to LVS inoculation. Organ weight change during infection was followed in both liver and spleen as an indication of infection. Size changes suggest the degree of gross pathologic change which, by their magnitudes, help relate LVS infection among groups of mice. Figure 2A and B are representative of four experiments with six or seven mouse organs for each data point. The greatest weight changes were on day 11 after LVS inoculation. Increase in organ weight in i.v. inoculated mice consistently preceded that in s.c. inoculated mice, but maximum weights were similar and decreases of weights occurred at similar rates. Weight change in spleen correlated directly with changes in numbers of nucleated cells.

Incorporation of spleen size changes into modulation data. Most of the spleen size change during LVS infection is due to cellular infiltration. Figure 3 consists of data collected as described for Table 1 and Figure 1A and B. The only difference is that nucleated cell numbers/spleen have been incorporated into the display. As with Figure 1A and B, three immunomodulatory phases are evident. However, the suppression phase appears more as a loss of augmentation rather than severe suppression.

Bacterial recovery from blood, liver and spleen. Figure 4 represents numbers of experiments and sizes of groups of mice as in Figure 2. Figure 4 shows that, in general, greater numbers of bacteria were recovered from liver than from spleen. In all cases, recovery of bacteria from blood was sporadic and limited to those mice with high numbers of organisms in their spleens and liver. Bacteria do not appear before day 3 in the spleens of s.c. inoculated mice.

DISCUSSION

The use of the primary in vivo immune response to SRBC to measure immunomodulation offers several unique possibilities. First, SRBC is a nonproliferating antigen and, therefore, delivered antigen dose can be easily controlled. Second, SRBC is unrelated antigenically to the LVS antigens and represents a distinct immune response. Third, results are assayable by the Jerne hemolysis in gel technique for specific antibody production. Fourth, SRBC is a T-cell recognition-dependent antigen which is representative of a wide variety of common antigenic stimuli. Fifth, T-cells and macrophages, known to play significant roles in the immune response to intracellular facultative bacterial infection, are also necessary in the sequelae leading to antibody production to SRBC. And, lastly, an in vitro system exists (Mishell-Dutton primary in vitro immunization) which allows numerous ways to dissect problems and observations in vivo, in order to elucidate immune response mechanisms.

The immunomodulation in s.c. LVS-inoculated mice can be described in three phases (Howell, H. M., and D. W. Seburn, Fed. Proc. 37:1847, 1978) and can be related to splenomegaly. These are: augmentation before noticeable splenomegaly, suppression coinciding with the period

of most rapid increase in spleen size and maximum enlargement, and a later phase of augmentation coinciding with the slow decrease in spleen size.

Spleen size is closely related to presence of bacteria within it. This is most clearly shown by comparing spleen size and numbers of bacteria, as shown in Figures 2 and 4. Moreover, F. tularensis was recovered from the spleens of i.v. inoculated mice from day 1, while this bacterium was not recovered from spleens of s.c. inoculated mice until day 3. Splenomegaly is consistently evident in mouse spleens yielding bacteria.

The lack of splenic sequestered bacteria among s.c. LVS inoculated mice throughout days 1 and 2 suggests control of proliferation or of transport of bacteria to the spleen. It is obvious from the magnitude of bacteria first recovered from s.c. inoculated mouse spleens at day 3 (Fig. 4) that bacterial proliferation has been ongoing. Since no bacteria were recovered from spleen, liver or blood, it appears that they are localized at the site of inoculation. Pathologic examination of these mice showed lesions on abdominal musculature in s.c. inoculated mice, which were not found in i.v. inoculated mice. Moreover, a small granulomatous mass is always formed intradermally near the site of inoculation. It is probable that the bacteria are contained by local tissue involvement and the sieving effect of the immediate inguinal lymph node. However, some communication of nonspecific immunity to splenic populations is suggested by the early augmentation phase.

Blood-borne bacteria are rarely recovered from mice at the low-level inoculum used. In the rare cases of this recovery, it has been noted that it correlates well with mice carrying massive numbers of bacteria in their

spleens and livers. Apparently a level of bacteria exists, above which the liver and spleen are no longer capable of filtering them from the circulation.

Peak splenic bacterial recovery was on day 5 postinoculation (Fig. 4). Numbers of viable bacteria declined rapidly until recovery was limited to few bacteria in random mice after day 9. Spleen size peaked approximately on day 9. This is probably due to the fact that no further bacteria are being added to the mass in the spleen and the bacteria killed, but trapped in granulomas, are removed slowly from these sites. Pathologic evidence supports slow resolution of the granulomas, as does the slow decrease in total spleen size over the subsequent 2 to 3 weeks.

The fact that the modulatory effects occur over a 30-day postinoculation time span suggests that the causes are not merely due to an effect of viable bacteria. Viable bacteria may play a role in the early modulation and, perhaps, in the suppression, but have virtually disappeared by day 9 (Fig. 1A). Further, the fact that day 5 augmentation occurs at peak bacterial recovery mitigates against this sort of direct pathologic effect. Instead the immunomodulatory effects might better be explained by changes in intercellular communication or regulatory phenomena.

If it is assumed that large masses, or at least rapidly increasing amounts, of bacterial antigen are responsible for suppression, and that stable or smaller masses of bacteria cause augmentation, an interesting observation can be made. Day 5 augmentation is clearly at a point of large bacterial mass. Reconciling these data to the above hypothesis can only be done by assuming that the critical point is early in the induction-production cycle. By allowing this assumption, all data

appear to fit this hypothesis. This is a particularly appealing rationale because: i) mechanisms during the first 2 days of the 5 days necessary to complete anti-SRBC antibody production are highly macrophage (adherent cell) dependent, and ii) macrophage populations play an important role in disposing of intracellular facultative bacteria.

Certain populations of regulatory cells have been reported to reside wholly or in large part in the spleen (9, 10, 15). The amount and proximity of bacteria to the splenic populations involved in primary immune induction to SRBC suggest possible involvement in development of immunoregulatory cell populations located in the spleen. Thus the macrophage may not be the only cell type whose function is affected by F. tularensis.

A further possible influence on immunomodulation may be nutritional deficiency. Subjective observations of mice ill during infection suggest that crouching, ruffled hair and weight loss can occur. Groups in which at least 20 to 25% of the mice show these effects also show greatest suppression. These effects are most pronounced on day 7 and, in most cases, are only identified with the utmost difficulty by one week after this severe period. The rare mice showing these effects persisting in a pronounced way by day 12 or 14 are invariably profoundly suppressed in the in vivo assay for anti-SRBC DPFC. Several reports have correlated protein deficiency and suppression (2, 5, 11, 15). Maximal weight loss has been shown to occur in the first 7 days of low-protein diet administration (12). This period corresponds to the period between LVS inoculation and maximum debilitation. Unfortunately, all tests of immune response ability in the literature were performed

well beyond this time and are not valid for direct correlation with our data.

Several phenomena including large antigen load, altered cellular interaction in enlarged organs, infectious factors, and nutritional imbalances, all appear at times near the maximal suppressive phase shown. Each has been associated with altered immune function. In vitro experiments are in progress in order to define cell population interactions during immunomodulation and particularly suppression.

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TABLE 1. Composite of seven 30-day experiments showing immunomodulation in vivo during LVS-produced infection in AKR/J male mice

Group	Mean PFC/10 ⁶ cells by day postinoculation						
	5	8	12	15	20	25	30
Normal mice	541 ± 15	552 ± 26	594 ± 13	608 ± 23	659 ± 29	637 ± 25	575 ± 19
Infected mice	638 ± 16	480 ± 14	354 ± 16	435 ± 34	600 ± 23	668 ± 32	679 ± 16
z (Infected/Normal)	117	83	59	71	91	104	118
P <	0.01	0.25	0.01	0.05	NS	NS	0.01

FIGURE LEGENDS

FIG. 1. Immunomodulation of in vivo immune response during LVS-induced infection. Shown are the three phases, augmentation, severe suppression and recovery with further augmentation. A. Total counts. B. Normalized plots as % of normal.

FIG. 2. Wet weight changes of tissues of i.v. and s.c. infected mice. A. Spleen; maximal weight change ranged between 200 and 300% of normal. B. Liver; maximal weight change approximately 150% of normal.

FIG. 3. Incorporation of spleen size into DFC data. Immunomodulation effects are seen, but augmentation does not correlate with spleen size.

FIG. 4. Recovery of colony-forming bacteria from spleen, liver, and blood from s.c. infected mice.

Fig. 1

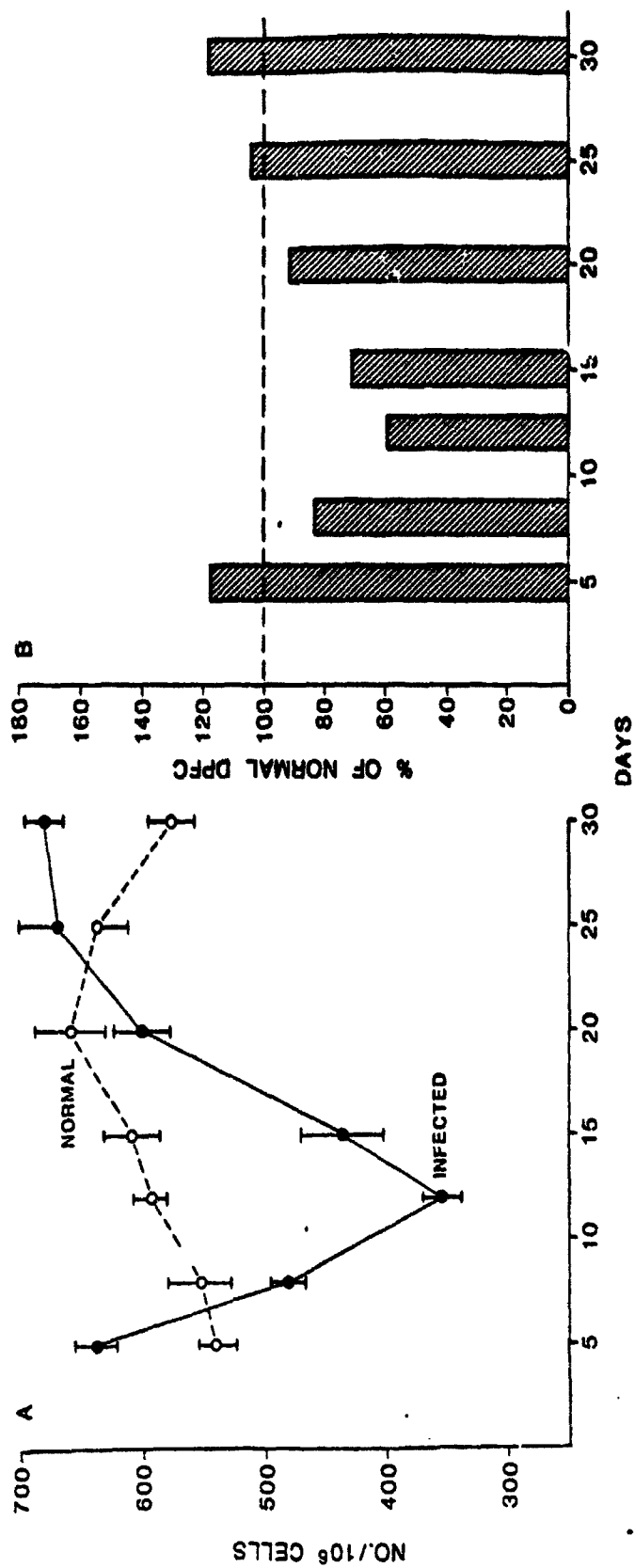


FIG. 2

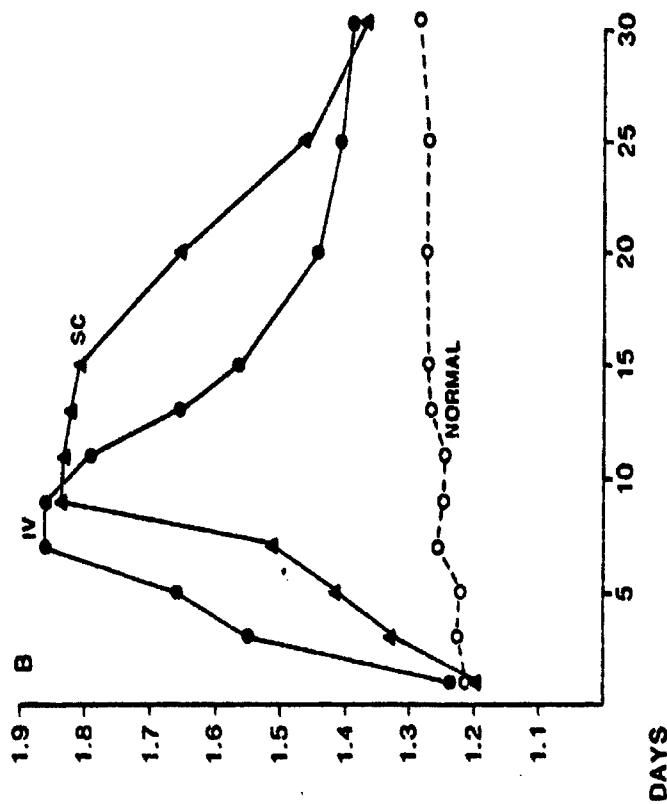
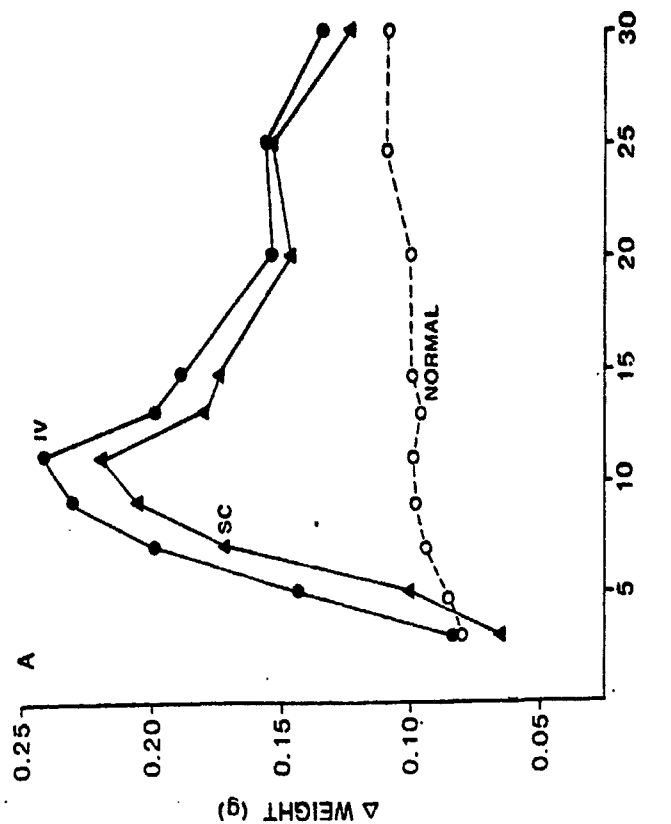


Fig. 3

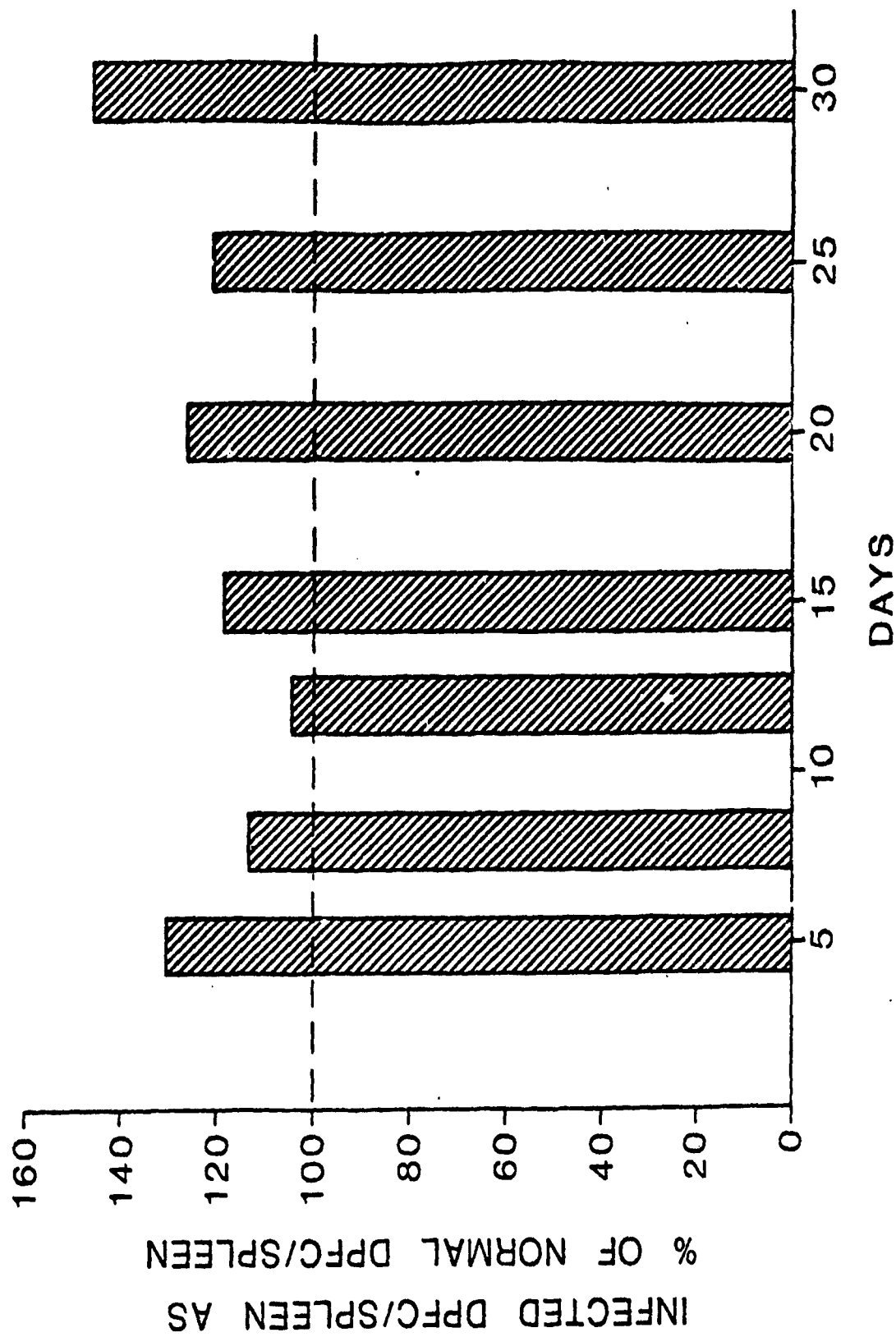


Fig. 4

