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Effect of Antigen Dose on Lymphatic Tissue Germinal Center Changes.* (30760)

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Earliest histologic changes observed during the immune response involve lymphatic tissue germinal centers, and various investigators have suggested that these centers are an integral part of the immune system(1-4). Three consistent histologic findings during the early period of spleen lymphatic tissue reaction to an antigenic stimulation of 1 ml of 10% sheep erythrocyte suspension have been reported(1,2). The first change is germinal center loss or dissociation; the second is the occurrence of large pyronin-staining cells throughout the lymphatic nodule and splenic red pulp. The presence and general significance of large pyronin-staining cells for reaction to antigenic material have been recognized for some time, and these cells are thought by many investigators to be the source of plasma cells and other immunologic phenomena(2,5,6). The third change is germinal center recovery, with hyperplasia beginning 2 days after antigen injection.

Autoradiographic and histologic studies have suggested that germinal center cells of spleen white pulp can be stimulated either directly or indirectly by antigen and that these stimulated cells emigrate from the centers to other areas of the spleen(7). This response ultimately results in what has been termed the dissociative growth of the center cells. These studies have also suggested that on a dynamic and morphologic basis, a correlation exists between germinal center cells and large pyronin-staining cells characteristically seen in lymphatic tissue during early intervals of the immune reaction.

Germinal center recovery with hyperplasia has been the most frequently described histologic alteration in lymphatic tissue during the immune response. The role of hyperplastic germinal centers, however, has not been resolved.

The purpose of this study was to investigate the effect of antigen dose on spleen germinal center changes and to correlate these changes with production of specific serum antibody during the first 30 days after primary antigenic stimulation. The results indicate a dose-response relationship between antigen dose and germinal center changes and a correlation between germinal center changes and specific serum antibody production.

Materials and methods. a) *Animals.* Male, $BC3F_1$ |(C3H/AN ♀ × C_{57} Bl ♂) F_1 Cum.| mice 13-16 weeks of age were used. They were kept 10 to a cage and given food and water *ad libitum.*

b) *Antigen.* Sheep blood was obtained fresh in modified Alsever's solution(8) and

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washed in phosphate-buffered isotonic saline (pH 7.1) by alternate suspension and centrifugation at 700 × *g*. Finally, 0.01, 0.1, 1.0, 10.0 and 25.0% suspensions of erythrocytes (SRBC) were made in phosphate-buffered isotonic saline. Immunization was carried out by intravenous injection.

c) *Procedure.* Mice were randomly divided into 6 groups of 60 animals each. All mice in Group 1 were injected intravenously with 1 ml of 0.01% SRBC. Animals in Groups 2, 3, 4 and 5 were similarly injected with 0.10, 1.0, 10.0 and 25.0% doses respectively. Animals in Group 6 were non-treated controls. Five mice from each group were killed by severing the aortic arch at 2 hours and days 1, 2, 3, 4, 6, 8, 12, 16, 20, 24 and 30 after antigen.

d) *Serology.* Blood was collected from each mouse. Serum was separated after standing overnight at 2 to 5°C and then stored at −20°C until titrated.

Serial 2-fold dilutions of serum in phosphate-buffered saline were made in volumes of 0.2 ml; to each dilution was added 0.05 ml of a 1% suspension of SRBC, and the tubes were incubated for 1 hour at 37°C and for 16 hours at room temperature. The $\log_2$ titer was determined as the reciprocal of the highest dilution showing hemagglutinin.

e) *Tissue preparation and planimetry.* The spleen was removed from each mouse, weighed and fixed in Bouin's fluid. Longitudinal sections were taken from the central region of the spleen and stained with hematoxylin and eosin.

For the determinations of surface areas of the white pulp germinal centers in each longitudinal spleen section, the image of the spleen section was microprojected at a magnification of 120×. The outline of each germinal center in a longitudinal spleen section was traced on paper and the respective areas were measured with a compensating polar planimeter. At each interval the germinal center areas were plotted graphically as the mean germinal center surface area per longitudinal spleen section. One longitudinal section was measured from each of the 5 animals killed at each interval. In previous studies it has been shown that if spleen weight is constant the mean number of centers per longitudinal spleen section corresponds to the mean center volume(1). This correspondence could be altered by changes in spleen weight. Therefore, the measurement of the number of germinal centers per longitudinal spleen section is a relative value dependent on the size of the center and overall size of the spleen. Thus, the primary parameter considered in the present study was the mean germinal center size as reflected by mean surface area.

Results. Germinal center changes and hemagglutinin titer in mice injected intravenously with 1 ml of 0.01% sheep erythrocytes are presented in Fig. 1. At this dose there is a 4-day latent period during which no increase in serum hemagglutinin can be detected. The hemagglutinin titer rises between days 4 and 6 and decreases through day 30 after antigen.

During this latent period there is a mild hyperplasia of the germinal centers at day 1, followed by a decrease in germinal center size. Between days 4 and 6, a marked germinal center hyperplasia occurs which corresponds to the log phase of the hemagglutinin titer. The germinal center hyperplasia declines between days 8 and 30.

There were no significant differences in spleen weight in this group, at any interval, in comparison to the non-treated control animals.

In mice injected with 0.1% SRBC suspension, a 2-day latent period occurred with peak hemagglutinin titer reached 6 days after antigen (Fig. 2). A decreasing titer was measured between days 6 and 30. Germinal center measurement demonstrated a cyclic atrophy during the first 3 days, followed by an approximate doubling of mean surface area at day 6. Germinal center surface area decreased between days 6 and 30, corresponding with the decreasing hemagglutinin titer. Normal center surface areas were measured at day 20 after antigen.

No significant difference in spleen weight was recorded in this group at any interval in comparison to the non-treated control animals.

An approximately 2-day latent period in hemagglutinin titer was measured in mice re-

ceiving 1.0% SRBC, with peak titer seen at 5 days after antigen. Titer was maintained at a high level throughout the duration of the experiment (Fig. 3).

A marked decrease in germinal center size was noted at 1 and 2 days after antigen, with recovery and hyperplasia reaching a maximum at day 6. The growth phase of germinal centers lagged behind the log phase of antibody response by approximately 1 day. Although hyperplasia decreased between days 6 and 8, the germinal centers remained hyperplastic until day 20, with return to normal levels at 24 days. The sustained hyperplasia correlated with the sustained hemagglutinin titer until day 20.

A 27% increase in the ratio of spleen weight to body weight was observed in the mice at 3 days after antigen. Spleen weights returned to normal levels at day 5.

The hemagglutinin profile with 1 ml of 10% SRBC approximated the profile when

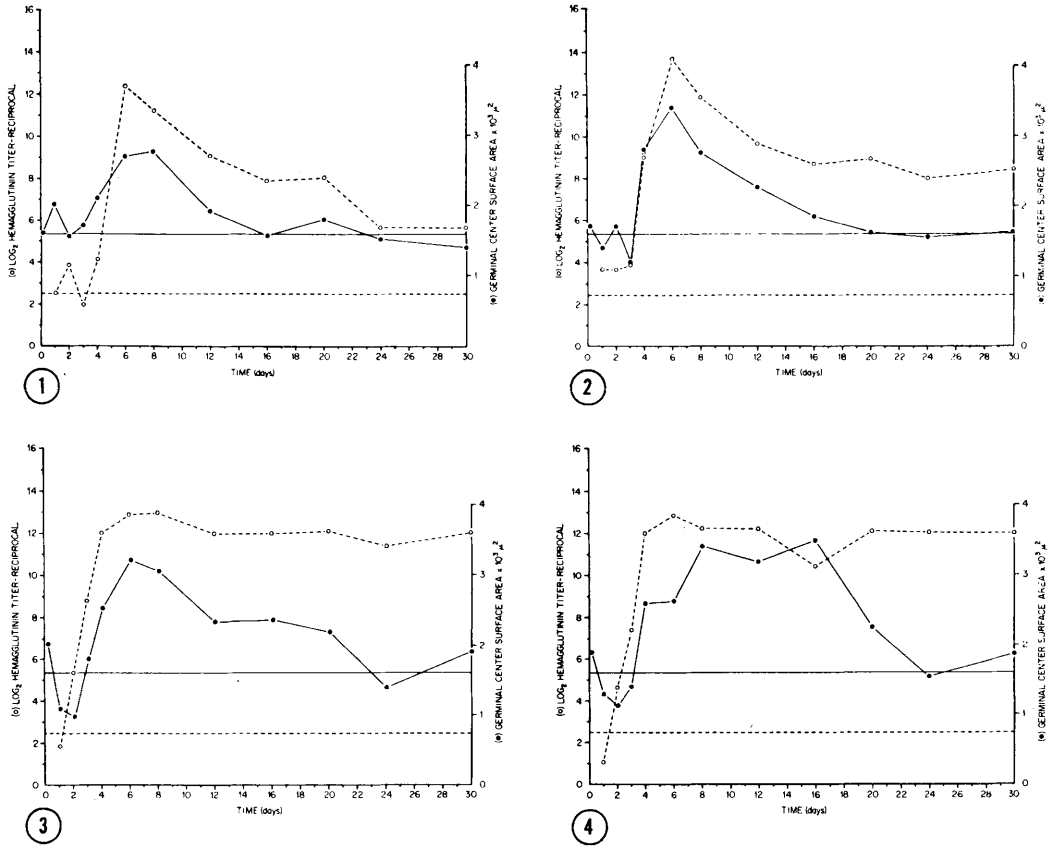

FIG. 1. Germinal center changes and serum hemagglutinin production in mice injected with 1 ml of 0.01% SRBC: ●——● mean germinal center surface area × 10^3 μ^2, ○– – –○ $\log_2$ HA titer reciprocal, ——— germinal center measurements in noninjected control mice, – – – – hemagglutinin titer in noninjected control mice.

FIG. 2. Germinal center changes and serum hemagglutinin production in mice injected with 1 ml of 0.1% SRBC: ●——● mean germinal center surface area × 10^3 μ^2, ○– – –○ $\log_2$ HA titer reciprocal, ——— germinal center measurements in noninjected control mice, – – – – hemagglutinin titer in noninjected control mice.

FIG. 3. Germinal center changes and serum hemagglutinin production in mice injected with 1 ml of 1.0% SRBC: ●——● mean germinal center surface area × 10^3 μ^2, ○– – –○ $\log_2$ HA titer reciprocal, ——— germinal center measurements in noninjected control mice, – – – – hemagglutinin titer in noninjected control mice.

FIG. 4. Germinal center changes and serum hemagglutinin production in mice injected with 1 ml of 10.0% SRBC: ●——● mean germinal center surface area × 10^3 μ^2, ○– – –○ $\log_2$ HA titer reciprocal, ——— germinal center measurements in noninjected control mice, – – – – hemagglutinin titer in noninjected control mice.

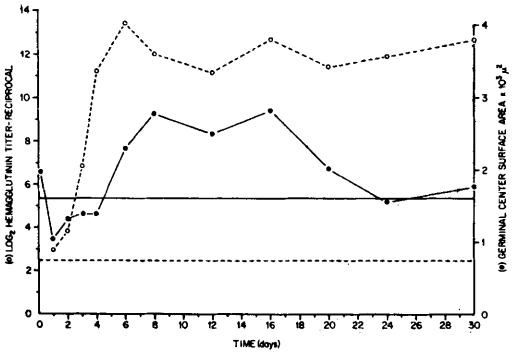

FIG. 5. Germinal center changes and serum hemagglutinin production in mice injected with 1 ml of 25.0% SRBC: ●——● mean germinal center surface area × 10^3 μ^2, O - - - O $\log_2$ titer reciprocal, ——— germinal center measurements in noninjected control mice, - - - - hemagglutinin titer in noninjected control mice.

1% SRBC was used as antigen (Fig. 4). Measurement of germinal center surface area indicated a marked atrophy to day 3, with hyperplasia being established by day 8. The growth phase of germinal centers lagged behind the hemagglutinin log phase by approximately 2 days. Germinal center hyperplasia was maintained until day 20. Persistent germinal center hyperplasia at this dose corresponds with elevated hemagglutinin titer until about day 20.

Spleen weights in this group of mice increased, resulting in a 36% change in the ratio of spleen weight to body weight at day 3, with normal spleen weights being measured by day 6 after antigen.

In mice injected with 1 ml of a 25% SRBC suspension, hemagglutinin titer was not significantly different from the 1% or 10% dose of antigen (Fig. 5).

A marked germinal center atrophy was measured between days 1 and 4, with recovery and hyperplasia at day 8 after antigen. The growth phase of germinal centers lagged behind the hemagglutinin log phase by approximately 3 days. Hyperplastic germinal centers persisted to day 20. Germinal center hyperplasia was less with 25% SRBC than that seen at lower antigen doses. The germinal center atrophy with the 25% dose was the greatest seen in these experiments.

A marked increase in spleen weight was recorded in these mice between days 2 and 6 after antigen. A 34% maximum increase in the ratio of spleen weight to body weight was measured at day 2. Spleen weights returned to normal levels at day 8.

Discussion. Many investigators have considered the effect of antigen dose on serum antibody production (see 9 for literature). However, the effect of antigen dose on cellular changes (in intact animals) has not been systematically investigated.

In recent autoradiographic studies of mouse spleen, evidence was presented that suggested germinal centers in normal mice are in a steady state in which cells are entering and leaving each compartment at a constant rate (7), a finding also described in rat spleen by Fliedner *et al*(10). These data(7) further show that a 1 ml intravenous dose of sheep erythrocytes results in a disruption of the steady state, with an increased number of dividing cells and an increase in the number of cells migrating from the germinal centers into the associated lymphocyte mass and red pulp. This has been termed germinal center dissociation, resulting in the dissociative growth of germinal center cells outside of the centers. This results in a temporary atrophy or loss of the pre-existing germinal centers.

Results of the present study indicate that dissociation or loss of germinal centers during the first 24 hours after SRBC antigen is dose dependent, with a direct relation existing between duration of germinal center loss or atrophy and increasing antigen dose. Atrophy is achieved only at higher antigen doses which are capable of drastically altering the flux of cells from the centers. In this study an antigen dose that the germinal centers respond to by hyperplasia alone appears to be approached with the 0.01% SRBC suspension.

Several investigations concerned with changes in lymphoid tissue during antibody formation have failed to detect the germinal center loss or atrophy while still describing the hyperplasia. These studies have either used low doses of soluble antigens or failed to study changes during the first 24 hours (3,4,11). Based on present work this would imply that the antigen type and dose were incapable of altering the flux of cells from the centers to the point that there was a resulting atrophy, or that the dissociation was

missed due to experimental procedure.

The most consistently described change in splenic germinal centers after antigen stimulation is hyperplasia. There is a correlation between the degree of germinal center hyperplasia and serum hemagglutinin production. At the lower antigen doses (0.01 and 0.1% SRBC) the degree and duration of hyperplasia are proportional to the serum hemagglutinin titer. At greater antigen doses (1.0 to 25.0%), where high titers were maintained, the hyperplasia is of a sustained nature; however, at each of these doses the growth phase of germinal centers trails the log phase of the titer. The response of increasing germinal center hyperplasia with increasing antigen dose fails at the 25% dose. It can be suggested that the amount of antigen is beyond the limits of the splenic capacity to react and that other secondary physiologic factors are involved. Thus, the measurement of spleen germinal centers changes alone is not adequate for assaying this large dose.

In addition, other lymphatic tissues besides the spleen might respond to higher antigen doses giving rise to serum antibody when the splenic germinal centers have returned to normal levels. At the lowest antigen doses, the spleen might be the only significant source of serum antibody. Further study is required to elucidate these points.

The role of the hyperplastic germinal centers in the immune response and their relationship to the serum hemagglutinin is an important question. White(12), using fluorescent tagged antibody in chickens and rabbits, observed specific antibody production in these hyperplastic centers during the later intervals of the secondary immune response. This finding has also been investigated by Ortega and Mellors(13) and Mellors and Korngold(14). Whether a source of the sustained serum antibody is the cells of the germinal centers is still not resolved.

These data suggest that at higher antigen doses it is unlikely that the hyperplasia plays any major role in early serum hemagglutinin production since the growth phase of the centers consistently lags behind the log phase of the titers. During this early period the loss of centers or dissociation of center cells seems to be the critical change. Autoradiographic studies have been presented which suggest that dissociated germinal center cells may be a source of plasma cells(1).

Germinal center cells have also been implicated as memory cells for secondary antigenic response(12). The hyperplasia measured at the later intervals of the immune response might be more closely associated with this function of the centers.

Summary. Investigations were performed on the effect of various doses of sheep erythrocyte antigen on spleen germinal center changes. These changes were correlated with the production of specific serum antibody during the first 30 days after primary antigen stimulation. The results indicate a dose-response relationship between antigen dose and germinal center changes. A correlation was observed between the germinal center changes and specific serum antibody production.

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