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Requirement for Continuous Antigenic Stimulation in the Development and Differentiation of Antibody-Forming Cells: Effect of Antigen Dose

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Summary. The concept that antigen has a continuous role in the recruitment and differentiation of immune progenitor cells was tested with optimum and suboptimum doses of heterologous erythrocytes in mice. These studies further evaluated an immune cell maturation scheme in which continuous antigenic stimulation is required for both the recruitment of 'antigen-sensitive units' and the expansive proliferation of a distinct sensitized cell compartment, which undergoes irreversible differentiation to functional antibody-forming cells. Haemolytic plaque-forming cell capacity during both the primary and secondary immune reactions were studied, both in the intact animal and with the spleen cell transfer technique. This *in vivo* culture technique was used to measure the sensitized cell compartment in the absence of existing antibody regulatory mechanisms. The results clearly demonstrate a higher detectable secondary immune capacity in the suboptimum antigen dose group than in the optimum antigen dose group. This was demonstrated for both the 19S and 7S cellular responses, as well as with humoral antibody levels measured in the spleen cell recipient mice. It can be concluded that in the presence of a suboptimum dose of antigen, which rapidly diminishes during the early intervals of the primary response, there is adequate recruitment with subsequent preservation or rescue from antigen-mediated depletion of the sensitized cell compartment, at the expense of the detectable primary response.

INTRODUCTION

The concept that antigen has a continuous role in the recruitment and differentiation of immune progenitor cells has not only a functional basis but, in our estimation, a structural one as well. This morphologic basis rests on the observation, during the primary response, of extracellular localization and persistence of antigens and/or antigen-antibody complexes on unique dendritic reticular cells of lymphatic tissue germinal centres (Nossal, Abbot, Mitchell and Lummus, 1968; Szakal and Hanna, 1968; Hanna and Szakal, 1968). A strong argument can be made for interdependency between antigen localization and sustained proliferation of the immunocompetent cells in these follicular, thymus-independent regions of lymphatic tissue (Nossal, Ada and Austin, 1965; Hanna, Nettesheim and Walburg, 1969).

In terms of functional aspects of immunity, the strongest support for this concept is derived from recent studies of passive antibody-mediated immune suppression (Moller

and Wigzell, 1965; Wigzell, 1966; Uhr and Moller, 1968). This type of suppression has been viewed mainly as an inhibition of recruitment of immunocompetent progenitor cells resulting from antigen–antibody interaction and the inactivation of antigen. The immune cell maturation scheme of Sercarz and Coons (1962) and as used by Sterzl (1967), commonly referred to as X-Y-Z model, is based on the requirement of continuous antigenic stimulation in the development and differentiation of antibody-producing cells. As defined, the antigen-sensitive cell (X-cell) is converted to sensitized Y-cells upon stimulation. Recent evidence suggests that this step would require interaction with at least one other cell type (Miller and Mitchell, 1968; Mosier, 1969). Thus, what is actually involved is an antigen-sensitive unit (Shearer, Cudkowicz, Connell and Priore, 1968). Presumably, the Y-cell is not an efficient antibody-producing cell but acts as a sensitized progenitor cell that is qualitatively distinct from the antigen-sensitive cell of unprimed animals. Stimulation of the Y-cell by antigen results in proliferation of these sensitized cells, which expands the cell compartment prior to irreversible maturation of Y-cells to functional antibody-producing Z-cells.

More recently, in support of this immune cell model, Byers and Sercarz (1968) suggested that a condition of excess antigen depletes or depresses the Y-cell compartment. We have demonstrated in the *in vivo* transfer system that after initial recruitment of antigen-sensitive cells, passive administration of antibody results in an enlarged Y-cell compartment (Hanna, Nettesheim and Francis, 1969). We considered this enlarged sensitized cell compartment to be a possible result of the rescue of Y-cells from their normal antigen-driven maturation to functional antibody-forming cells, which is a natural occurrence during the primary response.

One characteristic we would expect to observe during a primary antibody response in which high levels of immunogen are available is a rapid transformation of the sensitized progenitor cells (Y-cells) to functional cells (Z-cells). This condition would favour a primary antibody response rather than the establishment of secondary antibody-forming potential. Conversely, low levels of antigen would favour preservation of the sensitized cell compartment (Y-cell) rather than production of the antibody-producing cell (Z-cell).

There is experimental evidence supporting both possibilities. Results of several studies using a minimal antigen dose have demonstrated the development of a good secondary antibody-forming potential with little or no detectable primary response (Salvin and Smith, 1964; McDevitt, Askonas, Humphrey, Schecter and Sela, 1966; Hanna, Makinodan and Fisher, 1967). It has been observed that high initial doses of some antigens produce an optimum primary response, but subsequent boosting gives a poorer secondary response than the one produced in animals primed with a lower initial dose and given an equivalent boosting dose (Uhr, Finkelstein and Bauman, 1962; Sevhag and Mandel, 1964). Thus, antigen dose is important in controlling the character of the immune response. However, one can still question the mechanism by which this is accomplished. The depression of sensitized cells due to an antigen dose effect may be a consequence of 'exhaustion' of sensitized cells or a result of specific antibody-mediated immune suppression in the intact animals, the latter being either an effect on priming and/or a result of the higher pre-existing antibody levels at the time of secondary challenge.

The purpose of this study was to evaluate the effect of antigen dose on the concept of sensitized cell exhaustion during the primary immune response. Haemolytic plaque-forming cell capacity during both primary and secondary immune reactions was studied, both in the intact animal and with a spleen cell transfer technique.

MATERIALS AND METHODS

Animals

Male BC3F$_1$ (C57BL/6 females × C3H/An males)F$_1$ specific-pathogen-free (SPF) mice, 10–14 weeks of age, were used in these experiments. They were maintained ten to a cage and allowed free access to food and water.

Sensitization

Sheep erythrocytes (SRBC) were obtained sterile in Alsever's solution (Baltimore Biological Company, Baltimore, Md), washed three times in cold saline, and resuspended in cold saline to give the desired concentrations. All antigen injections were intravenous (i.v.) in volumes of 1 ml.

Haemolytic plaque-forming cells (PFC)

The PFC technique was used essentially as described by Jerne, Nordin and Henry (1963). An aliquot from the spleen cell suspension was mixed at 50° in 2·0 ml of melted 0·7 per cent Bacto-Agar in basal Eagle's medium containing 1 mg of diethylaminoethyl (DEAE) dextran and freshly washed sheep erythrocytes. The mixture was poured into a Petri dish containing a layer of Bacto-Agar in basal Eagle's medium. After incubation at 37° for 1 hour, 1·5 ml of frozen guinea-pig serum preabsorbed with SRBC diluted 1:10 (guinea-pig complement preparation) was poured on to the plate. At the conclusion of a second incubation period of 1 hour, direct plaques of haemolysis could be seen in the erythrocyte field.

A similar series of plates made from the same spleen preparation was incubated for 1 hour at 37°, after which 1·5 ml of a 1:100 preparation of goat anti-mouse γ-globulin serum (Hyland Laboratories, Los Angeles, Calif.) was added to each plate. The goat anti-mouse γ-globulin serum used in these studies was shown in our laboratory to inhibit 90 per cent of direct 19S plaques. The plates were then incubated for another hour, rinsed with basal Eagle's medium and supplemented with 1·5 ml guinea-pig complement preparation. After further incubation for 1 hour, indirect plaques were observed. Estimates of the number of indirect plaques were adjusted by subtracting 10 per cent of the number of direct plaques per donor spleen not inhibited by the goat anti-mouse γ-globulin.

In accordance with Plotz, Talal and Asofsky, 1968, we considered direct plaque-forming units to be 19S-haemolysin-forming units or cells (DPFC). Within the limitations described in the above study, we further considered indirect plaques to be primarily 7S-haemolysin-producing units or cells (IPFC). Generally, duplicate plates were made for each sample, and all plates were stained with 2 per cent benzidine.

The in vivo *culture system*

The system of spleen cell transfer was used as described previously (Hanna, Nettesheim and Francis, 1969). Prospective spleen cell donors were killed by exsanguination; the spleens were removed, homogenized by hand and the cells suspended in Hanks's solution. Aliquots of 25×10^6 spleen cells were transferred intravenously with 25×10^7 SRBC into lethally X-irradiated (850 rads) syngeneic recipients within 6 hours of removal of the donor spleens. At various times thereafter recipients were killed and bled for serum; the spleens were then removed, homogenized, and prepared for PFC determinations.

Serum haemolysins, and haemagglutinins

Individual serum samples were obtained and titrated for SRBC haemagglutinin in disposable plastic plates by use of a Cooke Microtitrator (Cooke Engineering Company, Alexandria, Va.) and a standard 2-fold dilution technique. Veronal buffer, pH 7·2, containing 0·1 per cent gelatin was used as diluant. The plates were read after 2–3 hours incubation at room temperature. Haemolysin activity (last well showing definite haemolysis) was then determined in the same plates by adding diluted guinea-pig-complement preparation (Flow Laboratories, Rockville, Md) and incubating the plates after resuspending the SRBC with an automatic shaker for 1 hour at 37°. To determine the relative amount of 2-mercaptoethanol (2-ME) resistant haemagglutinin or haemolysin, serum was incubated in modified veronal buffer containing 0·1 per cent gelatin and 0·1M 2-ME for 1 hour at 37°. Samples were then titrated for haemagglutinin and haemolysin as described above. The 2-ME-resistant antibody was assumed to represent haemagglutinin or haemolysin with a sedimentation rate mainly of 7S. The difference (total antibody activity minus 7S antibody activity) is considered to include the heavy or 19S antibody.

RESULTS

ANTIGEN DOSE EFFECT ON PRIMARY AND SECONDARY PFC RESPONSE IN INTACT MICE

Mice were injected with either 2×10^8 (1·0 per cent) or 2×10^6 (0·01 per cent) SRBC, and five animals from each group were killed at various times after antigen injection to measure the primary direct and indirect PFC responses and agglutinin and haemolysin titres. At 20 days the remaining mice were reinjected with SRBC doses equivalent to the primary antigen dose and the secondary immune response was measured in these animals.

As shown in Fig. 1, the primary DPFC response at 4 days is 60-fold greater at an antigen dose of 1·0 per cent SRBC than at one of 0·01 per cent. At peak response or the points thereafter, the difference is only about 2-fold. On the other hand, after secondary injection of the 0·01 per cent SRBC, DPFC response at days 3 and 5 is 6–9-fold higher than after the 1·0 per cent. The IPFC response in the 1·0 per cent group remained lower than the 0·01 per cent antigen dose group at all intervals studied.

A more dramatic effect was the weak IPFC response measured in mice challenged with 0·01 per cent SRBC (Fig. 2). At 5 and 8 days after challenge, the respective IPFC responses in this group of mice were about 40- to 20-fold lower than those in the group injected with 1·0 per cent SRBC. In contrast to this difference in the primary response, less than a 3-fold maximum difference between these two SRBC dosage groups was detected during the secondary response. However, less than 5000 IPFC per spleen separated these two dosage groups in terms of net excess IPFC (peak number of plaques detected during the secondary response minus peak number detected during the primary response).

Since there is a circulating antibody level at the time of secondary challenge, it was important to determine if antigen is a limiting factor in the expression of the secondary immune capacity in the intact animal. It was also important to ascertain whether the influence of antigen levels was greater on DPFC or IPFC expression. Mice primed with 0·01 per cent SRBC were subsequently rechallenged with 0·01 or 1·0 per cent SRBC at 20 days, and the DPFC and IPFC levels were compared at later intervals. An approximate 2-fold difference was measured in DPFC at day 3 after secondary challenge (Fig. 3). At

all other times the DPFC levels were comparable. At days 5, 6, and 10 the IPFC levels were markedly higher in the mice rechallenged with the 1·0 per cent SRBC dose. The difference was approximately 4-fold at peak IPFC response. In general, the effect of increasing the secondary challenge dose is greater on the IPFC response than on the DPFC response.

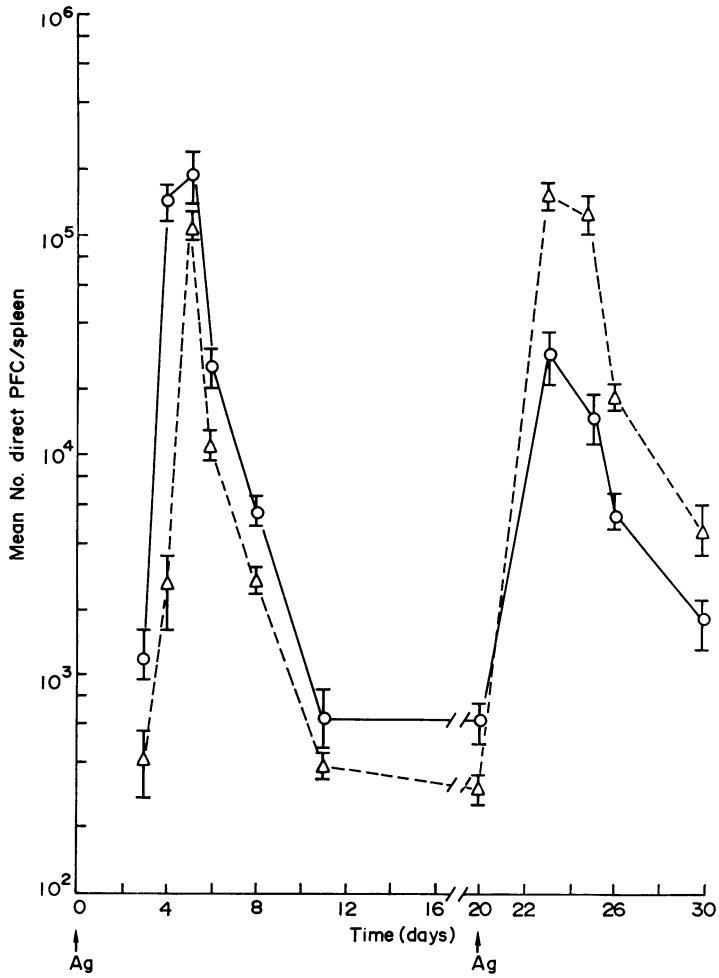

FIG. 1. Direct plaque-forming cell (DPFC) response after primary and secondary immunization at two antigen doses. ○, 2×10^8 SRBC (1 ml of 1·0 per cent SRBC); △, 2×10^6 SRBC (1 ml of 0·01 per cent SRBC).

EFFECT OF ANTIGEN DOSE ON SECONDARY IMMUNE CAPACITY AS MEASURED WITH THE *in vivo* CULTURE TECHNIQUE

In order to test the secondary immune capacity independently of regulatory mechanisms in the intact animal, we transferred 25×10^7 SRBC with 25×10^6 spleen cells from mice primed with 0·01 or 1·0 per cent SRBC into lethally irradiated syngeneic recipients. At 3, 5, 6, and 10 days after transfer, the recipient spleens were assayed for DPFC and

IPFC responses. At 5 and 6 days after transfer of spleen cells and SRBC, the DPFC response of the transferred spleen cells from donors primed with 0·01 per cent was about 20- to 30-fold higher than from the spleen cells of doners primed with 1·0 per cent SRBC (Fig. 4). The DPFC response of spleen cells from donors primed with the higher antigen dose was essentially the same as that acheived with 25×10^6 unprimed cells.

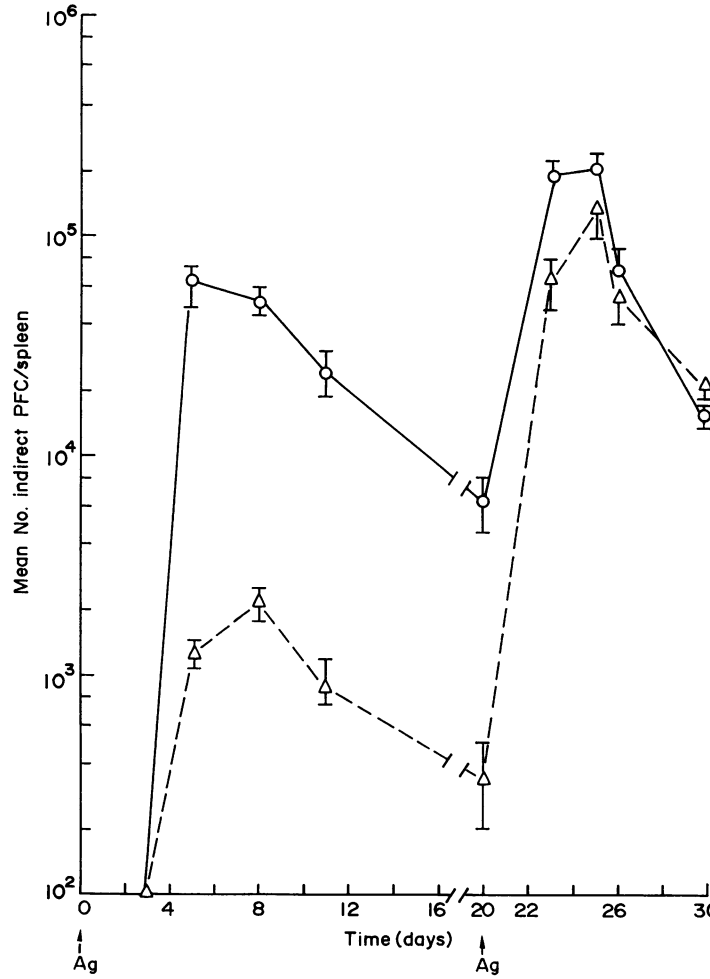

FIG. 2. Indirect plaque-forming cell (IPFC) response after primary and secondary immunization at two antigen doses. ○, 2×10^8 SRBC (1 ml of 1·0 per cent SRBC); △, 2×10^6 SRBC (1 ml of 0·01 per cent SRBC).

A similar difference between the two antigen dose groups was detected in IPFC (Fig. 5). At 5 and 6 days the IPFC response of spleen cells from donors primed with 0·01 per cent SRBC was 30- to 40-fold higher than the response obtained with an equivalent number of spleen cells from donors primed with 1·0 per cent SRBC. In this situation the IPFC response of the 1·0 per cent group was slightly higher at 5 and 6 days after transfer than that of the 25×10^6 unprimed spleen cells.

TABLE 1

SEROLOGICAL ASSAY OF SECONDARY IMMUNE CAPACITY IN IRRADIATED RECIPIENTS OF SPLEEN CELLS FROM MICE PRIMED WITH 0·01 PER CENT AND 1·0 PER CENT SRBC*

Time after transfer	Priming treatment	Total agglutinin†	2-ME-resistant agglutinin†	Total haemolysin†	2-ME-resistant haemolysin†
Days					
3	0·01% SRBC	0	0	0	0
	1·0% SRBC	0	0	0	0
	None	0	0	0	0
5	0.01% SRBC	9·0±0	7·5±1·5	7·4±0·6	3·6±0·9
	1·0% SRBC	7·8±0·4	7·6±0·5	—	—
	None	1·4±0·2	0	0	0
6	0·01% SRBC	9·8±0·2	9·4±9·4	9·4±0·6	8·0±0
	1·0% SRBC	9·0±0	9·0±0	6·2±0·2	5·4±0·2
	None	4·0±0·4	0	5·0±0·4	0·8±0·8
10	0·01% SRBC	11·6±0·2	11·6±0·2	11·0±0·5	10·6±0·2
	1·0% SRBC	13·0±0	13·0±0	9·0±0	9·0±0
	None	1·4±0·2	0	8·0±1·0	8·3±0·3

* Each group represents the mean of five mice ± one standard error of the mean.
† Reciprocals of $\log_2$ titre.

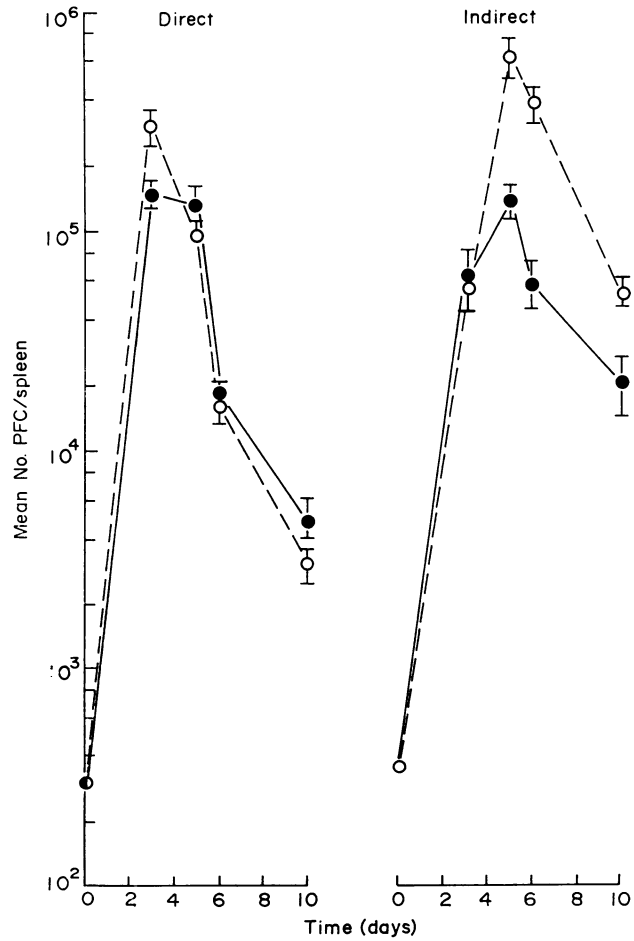

FIG. 3. Secondary direct (DPFC) and indirect plaque-forming cell (IPFC) response in animals primed with 2×10^6 SRBC and immunized for the secondary response with 2×10^6 SRBC (●) or 2×10^6 SRBC (○).

HAEMAGGLUTININ AND HAEMOLYSIN RESPONSE

The sera collected from the irradiated recipients were measured for haemagglutinin and haemolysin levels, with the results shown in Table 1. At days 5 and 6 the total agglutinin and haemolysin titres were higher in the recipients injected with spleen cells from the

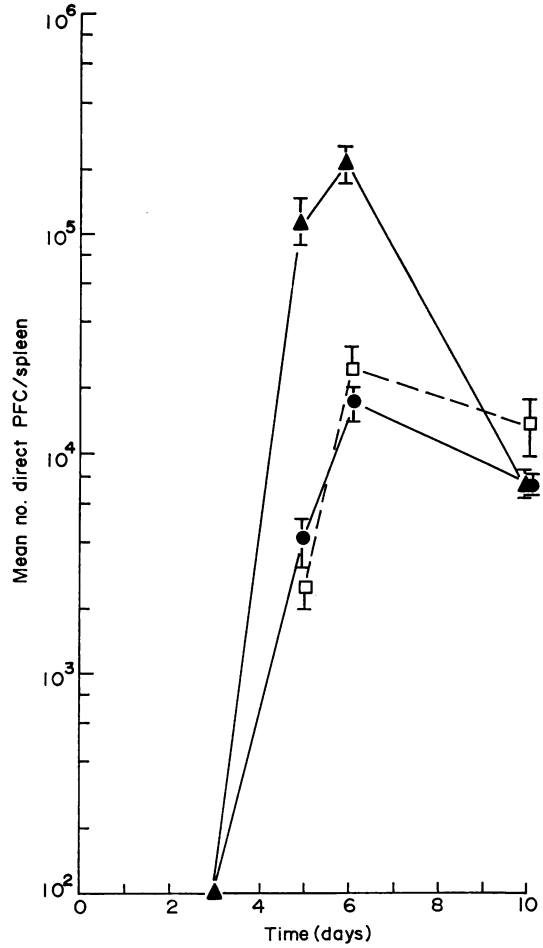

FIG. 4. Secondary direct plaque-forming cell (DPFC) response as measured from primed donors with the spleen cell transfer system. ●, Secondary response as measured in spleen cell (25×10^6) transfer, donors primed with 2×10^8 SRBC; ▲, secondary response as measured in spleen cell (25×10^6) transfer, donors primed with 2×10^6 SRBC; □, primary response as measured in spleen cell transfer of 25×10^6 normal spleen cells. All spleen cells were transferred with 25×10^7 SRBC.

0·01 per cent SRBC primed mice than in recipients injected with spleen cells from the 1·0 per cent SRBC primed mice. The controls are normal spleen cells. Except at day 10, no difference in 2-ME-resistant agglutinin could be detected between the two SRBC dosage groups. The 2-ME-resistant haemolysin level was significantly higher at days 6 and 10 in the 0·01 per cent SRBC dosage group. Although these results correlate well with the DPFC and IPC responses measured in the irradiated recipient spleen, the

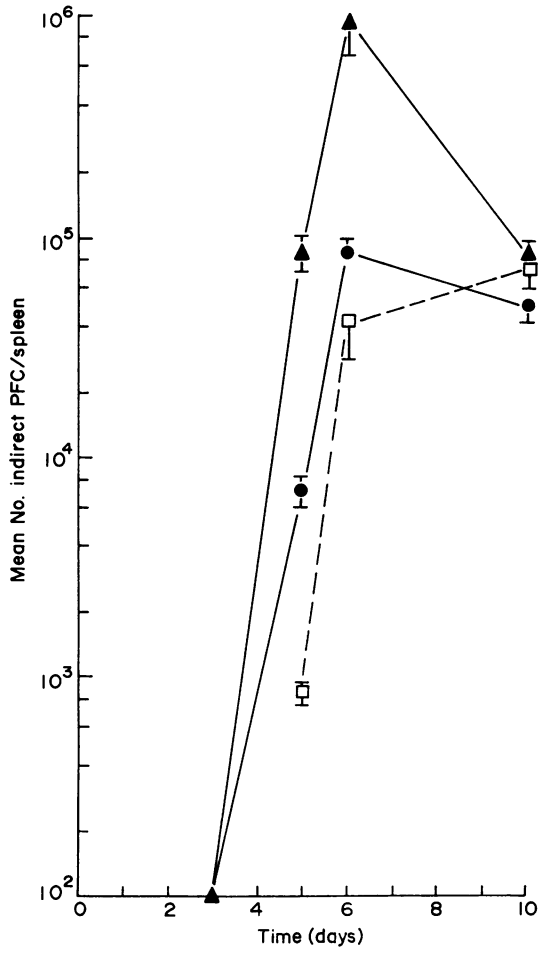

FIG. 5. Indirect plaque-forming cell (IPFC) response as measured from primed donors with the spleen cell transfer system. ●, Secondary response as measured in spleen cell (25×10^6) transfer, donors primed with 2×10^8 SRBC; ▲, secondary response as measured in spleen cell (25×10^6) transfer, donors primed with 2×10^6 SRBC; □, primary response as measured in spleen cell transfer of 25×10^6 normal spleen cells. All spleen cells were transferred with 25×10^6 SRBC.

magnitude of the difference between the two antigen dose groups is much smaller when measured by serum antibody.

DISCUSSION

These results, obtained by comparing the primary and secondary DPFC and IPFC responses of normal mice challenged with optimum and suboptimum SRBC doses, are in general agreement with previous studies (Uhr *et al.* 1962; Svehag and Mandel, 1964). It is clear that antigen dose markedly affects the character of the primary immune response, as well as the level of priming or secondary antibody-forming potential. With low doses of antigen (0·01 SRBC), minimal primary IPFC or 7S cellular response is achieved, but the

potential secondary capacity is comparable to that obtained with the high dose of antigen. The reverse occurs for the DPFC or 19S cellular response.

These experiments were designed to test an immune cell maturation hypothesis in which continuous antigenic stimulation is required for both recruitment of 'antigen-sensitive units' and proliferation and functional differentiation of the sensitized cell compartment.

Thus low doses of antigen, which rapidly diminish during the early phases of the primary response, would preserve or rescue the sensitized cell compartment from depletion. This would imply that at any time during the immune response the sensitized cell compartment has little if any regenerative potential (Nettesheim and Williams, 1968), and its size is entirely a function of antigen-induced recruitment *versus* exhaustive maturation of antibody-forming cells. Thus the two steps of immunocompetent cell differentiation should be dissociated in the presence of low or higher levels of antigen.

A major limitation of such interpretations of data in intact animals is the inability to control the variable effect of specific antibody regulation on cellular function (i.e. synthesis and secretion of antibody) and compartment size during the primary response, as well as the effect of persisting antibody on the secondary antigen dose. The results of this study clearly show that after priming with a lower dose of antigen, the detectable secondary 7S cellular response is significantly elevated, with a 100-fold increase in antigen. Thus the quantity of antigen and the concentration of antibody must play an important role in the expression of the secondary immune capacity in the intact animal.

We attempted to measure secondary immune capacity by spleen cell transfer in irradiated syngeneic recipients, which would provide an ideal *in vivo* culture for the immune reaction. Optimum antigen doses (ten SRBC to one dissociated spleen cell) for maximum stimulation of cells for both the high and low antigen dose priming groups were used. In this *in vivo* culture we tentatively dismiss consideration of antibody suppression of the response as a result of antibody-secreting cells in the inoculum for the following reasons: (1) no detectable serum antibody or significant numbers of PFCs could be measured in the recipients at 3 days after transfer. (2) It has been demonstrated that antibody-mediated immune suppression more severely affects the 19S cellular compartment and is less easily achieved with primed cell compartments (Uhr and Möller, 1968). In the present study the decreased memory cell function was observed in both the 19S and 7S primed cellular compartments with the latter being more severely depleted as a result of priming with high antigen doses. (3) In the primed cell transfer system, no significant difference with respect to expression of the primed cell compartment has been achieved with an antigen dose 10-fold lower than that used in the present experiment (Albright and Makinodan, 1965). We feel that this tends to rule out the effect of an early local antibody production in the recipient spleen that would decrease the level of stimulatory antigen. The results clearly demonstrate a higher secondary immune capacity of 25×10^6 spleen cells in the low antigen dose groups. This was demonstrated for both the 19S and 7S cellular responses, as well as for the humoral antibody response. On the basis of this procedure, and assuming that there is no undetected problem of antibody affinity, we may postulate the existence of significantly higher compartments of primed 19S and 7S cells in mice stimulated with 0·01 per cent SRBC compared to those stimulated with 1·0 per cent SRBC.

The immune cell maturation hypothesis on which the present studies are based, extends the role of antigen to continuously stimulating the intermediate sensitized cell compartment to produce functional antibody-forming cells. An important consideration, then, would be

the effect on the secondary immune response of blocking recruitment. We previously attempted to measure this effect by antigen competition (Hanna and Peters, 1970). Animals primed with doses of rat erythrocytes (RRBC), and challenged 2 days later with SRBC, showed a markedly suppressed secondary haemagglutinin response to RRBC, compared with controls. A possible but guarded interpretation of these results was that recruitment to RRBC occurs normally for 48 hours; then competition with the high concentration of SRBC successfully reduces recruitment to RRBC. Since no effect of competition on the amount of persisting antigen could be shown, it was concluded that the sensitized cell compartment was depleted as a result of maturation to antibody-producing cells during the primary response. This sensitized cell depletion was manifested as reduced secondary immune capacity.

Also, we have previously studied the effect of injecting isologous specific antibody on the primary and secondary response to SRBC (Hanna *et al.*, 1969). Mice were primed with optimum doses of SRBC in an attempt to induce maximum progenitor cell recruitment. Antigen was depleted by specific passive antibody at 1, 2, or 4 days after priming. These results demonstrated a depression of the primary antibody-producing cell compartment as measured in the intact mice and, subsequently, an enhancement of the sensitized cell compartment as measured in the spleen cell transfer system. These data suggested to us that although specific passive antibody is capable of blocking further recruitment of immunocompetent progenitor cells, it leads to maturation arrest of an established antigen-dependent, sensitized cell compartment.

In the light of these data and the interpretations presented in this discussion, an important morphological corollary is the existence of antigen depots in immunologically competent tissue. Intercellular antigen localization in plasma membrane infoldings of dendritic reticular cell within lymphoid germinal centres has been described in several studies (Hanna and Szakal, 1968; Nossal *et al.*, 1968; Szakal and Hanna, 1968). This correlates with intercellular fixation of specific antibody and the intense mitotic activity of parenchymal immunoblasts in these follicles (McDevitt *et al.*, 1966). It has also been demonstrated that both antibody-containing cells and extracellular antibody were present in some germinal centres, while other centres within the same organ remained negative throughout the period of observation. This was tentatively interpreted as an expression of the monospecificity of these structures with regard to antigen stimulating their development (Sordat, Sordat, Hess, Stoner and Cottier, 1970). We had previously made a similar suggestion based on patterns of antigen trapping in germinal centres of the spleen (Hanna, Peters and Francis, 1968). It must be pointed out that antigen localization in the *de novo* development of germinal centres appears to correlate more readily with the development of the 7S cellular response than with the 19S cellular response (Fitch, Stejskal and Rowley, 1969; Hanna *et al.*, 1969). It is not difficult to reconcile this requirement of the 7S response for antigen preservation on membranes, since major recruitment of the 7S cellular response occurs after the major sequestration of metabolizable antigen has occurred, whereas 19S recruitment occurs during the first 24 hours after injection, an interval of high levels of antigen throughout the lymphatic tissue (Sterzl, Johanovská and Milenová, 1969).

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