The Journal of Immunology

RESEARCH ARTICLE | APRIL 01 1980

The transient appearance of degenerate antibody early in the course of the immune response. FREE

E A Kan; ... et. al

J Immunol (1980) 124 (4): 1758–1762.
https://doi.org/10.4049/jimmunol.124.4.1758

0022-1767/80/1244-1758$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 4, April 1980
Printed in U.S.A.

THE TRANSIENT APPEARANCE OF DEGENERATE ANTIBODY EARLY IN THE COURSE OF THE IMMUNE RESPONSE[1]

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The appearance of degenerate antibody-secreting cells early in the course of the immune response was demonstrated. Mice immunized with SRBC had anti-SRBC plaques that were as much as 95% inhibitable by DNP-Ova or DNP-KLH, and mice immunized with DNP-KLH had anti-DNP plaques that were as much as 90% inhibitable by Fl-BGG. In addition, mice immunized with BRBC produced plaques detectable on TNP-SRBC. The data suggest that, early after antigen injection, antibody is produced that can react with both the immunizing antigen and with structurally unrelated antigenic determinants. The incidence of such degenerate antibody-secreting cells is maximal at 3 days after antigen injection and decreases thereafter. It is probable that the degenerate antibody is of very low affinity for the immunizing antigen.

Classically, the concept of strict specificity of antibody molecules and individual antibody-producing cells has been a central feature of immunologic theorization. Consistent with this view, the frequency of cells binding a given antigen has generally been reported to be very low, in the order of one in 10^5 cells (1–8). However, several examples of degeneracy in the immunologic system have been reported. For example, Secarz and coworkers (9, 10) found, that at saturating concentrations of antigen and conditions where capping of receptors does not occur, a remarkably high frequence of antigen-binding cells could be detected. As many as 4% of bone marrow cells bound an individual antigen and roughly 30% of cells binding one antigen also bound a second unrelated antigen. They suggested that there were different receptor "antibody" molecules on individual B cells, each receptor binding a single antigen. Another type of degeneracy was described by Richards and coworkers (11–14). These investigators identified individual antibody molecules (or myeloma proteins) that bound two unrelated ligands, in a competitive manner, at different subsites within the antigen-combining region. In addition, they showed that the production of such degenerate antibodies is favored by appropriate cross-boosting. Finally, Liacopoulos and co-workers (15–17) have demonstrated the existence of cells that appeared to secrete two distinct antibody molecules. The existence of a very high incidence of T cells that are specific for alloantigens of the major histocompatibility complex suggests the possibility of degeneracy of T cell specificities.

The significance of the high frequency of degenerate antigen-binding cells reported by DeLuca *et al.* (9, 10) has been unclear because it is not known whether the cells detected at high antigen concentration, which presumably have low affinity receptors, can actually be activated by antigen during an *in vivo* immune response. In the present study we have obtained evidence to support the concept that early in the *in vivo* immune response, a large number of antibody-secreting cells are present that appear to be degenerate with respect to antigen specificity and are probably producing antibody of very low affinity for the immunizing antigen. These degenerate antibody-secreting cells decrease in number with time after immunization.

MATERIALS AND METHODS

Antigens. Sheep red blood cells (SRBC) and burro red blood cells (BRBC)[3] were obtained from Flow Laboratories (Rockville, Md.). Ovalbumin (Ova) was obtained from Miles Laboratories (Elkhart, Ind.), keyhole limpet hemocyanin (KLH) from Pacific Biomarine Laboratories (Venice, Calif.), and bovine γ-globulin (BGG) from Pentex Division of Miles Laboratories (Kankakee, Ill.).

2,4-dinitrophenyl (DNP) derivatives of Ova and KLH were prepared (18) by the reaction of protein with 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals, Rochester, N. Y.). Fluorescein (Fl) isothiocyanate (FITC) was coupled to BGG according to Weir (19). Derivatives were purified by extensive dialysis. The concentration of the conjugated protein was determined by dry weight analysis and the degree of derivatization was estimated spectrophotometrically from the absorbance at 360 nm for DNP conjugates, and the absorbance at 429 nm (corrected for protein absorbance) for FITC conjugates. The DNP-Ova preparations used had approximately 15 DNP groups per Ova molecule. The DNP-KLH preparations had approximately 10 and 37 DNP groups per 100,000 m.w. The Fl-BGG preparations had approximately 22 Fl groups per BGG molecule. The degree of conjugation is indicated by a subscript.

Animals and immunization. Six- to 8-week-old male LAF_1 mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). They were immunized by the i.v. injection of 0.1 ml of a 5% suspension of washed SRBC or BRBC in normal saline, or

Received for publication October 9, 1979.
Accepted for publication December 26, 1979.
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[1] This work was supported in part by a research grant from the National Institutes of Health, United States Public Health Service, AI-11694.
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[3] Abbreviations used: BGG, bovine gamine globulin; BRBC, burro red blood cells; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl group; FITC, fluorescein isothiocyanate; Fl, fluorescein; KLH, keyhole limpet hemocyanin; Ova, ovalbumin; TNP, trinitrophenyl group; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

100 μg DNP_{10}-KLH in 0.5 ml PBS. Mice were sacrificed by cervical dislocation, at the time indicated, and their spleens were assayed for anti-SRBC, anti-BRBC, or anti-TNP plaque-forming cells (PFC).

Assay of PFC. Anti-SRBC, anti-BRBC, or anti-TNP PFC were determined by the method of Jerne *et al.* (20) as modified for slide assay by Dresser and Greaves (21). Washed SRBC or BRBC, or washed SRBC conjugated by the Rittenberg and Pratt method (22), with various concentrations of 2,4,6-trinitrobenzene sulfonic acid (TNBS) were used as target cells. Four, 20, and 40 mg TNBS/ml packed SRBC were used for preparing target cells (indicated by subscript). Slides were incubated for 1 hr at 37°C. Freshly frozen guinea pig serum (absorbed with 50% packed SRBC) was added, at a final dilution of 1:30, as a source of complement (C), and the slides were incubated for an additional 45 min. Indirect PFC were developed by addition of rabbit anti-mouse immunoglobulin antiserum, at a final dilution of 1:300. This inhibits approximately 60% of direct plaque formation. Indirect PFC are reported as total plaques observed in the presence of developing antiserum; the number of direct plaques was not subtracted. The effect, on plaque formation, of adding DNP_{15}-Ova, DNP_{37}-KLH, or Fl_{22}-BGG at (carrier) concentrations ranging from 1×10^{-9} M to 1×10^{-6} M, was determined.

RESULTS

Inhibition of anti-SRBC plaques by DNP-conjugates. Mice were immunized by the i.v. injection of 0.1 ml of 5% SRBC. Anti-SRBC PFC were assayed 3, 10, and 18 days after immunization. DNP_{15}-Ova and DNP_{37}-KLH were tested as inhibitors of plaque formation. It was found that anti-SRBC plaques can be inhibited by these DNP-conjugates. With time after immunization, the anti-SRBC plaques become progressively less inhibitable (Fig. 1). For example, at a concentration of 10^{-7} M, DNP_{37}-KLH inhibits 40% of the indirect anti-SRBC plaques present 3 days after immunization, whereas 10 days after immunization only 2% is inhibited. Direct PFC are inhibited to a greater extent than are indirect PFC at all times. It was found that incubation at 37°C, for 2.5 hours, in the presence of hapten-protein conjugates at 10^{-6} M, did not alter the viability of spleen cell suspensions when assayed by trypan blue dye exclusion. Thus, inhibition of plaque formation does not appear to result from simple death of spleen cells in the presence of high concentrations of the conjugates.

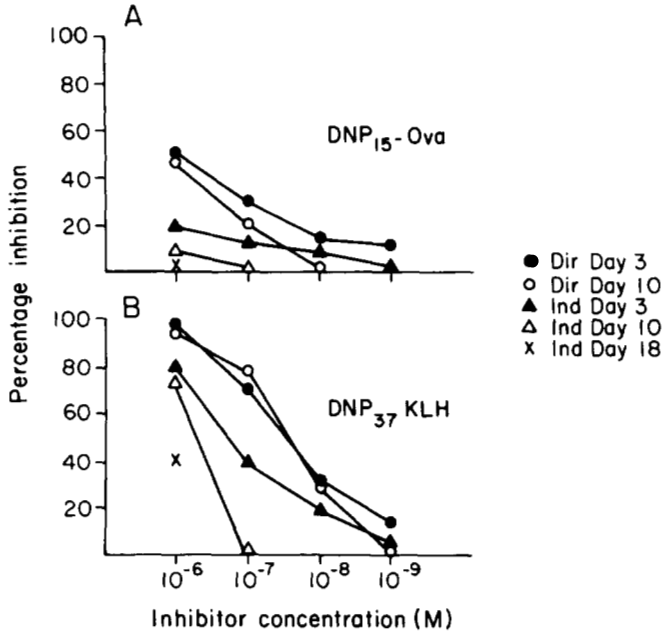

Figure 1. Inhibition of anti-SRBC plaques by DNP-conjugates. Mice were immunized by the i.v. injection of 0.1 ml of 5% SRBC and their spleens were assayed for direct (Dir) and indirect (Ind) anti-SRBC PFC 3, 10, and 18 days later. Panel A contains data on DNP_{15}-Ova as an inhibitor of anti-SRBC plaque formation and panel B contains data on DNP_{37}-KLH as an inhibitor. The percentage of inhibition of the number of plaques is indicated in the ordinate and the molar concentration of inhibitor is indicated on the abscissa.

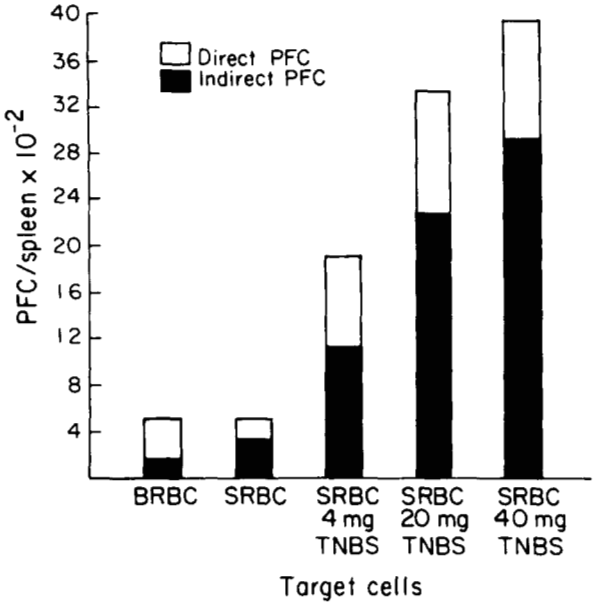

Figure 2. Anti-TNP PFC after immunization with BRBC. Mice were immunized by the i.v. injection of 0.1 ml of 5% BRBC. Three days later their spleens were assayed for direct and indirect PFC by using BRBC, SRBC, and SRBC that were coupled with the indicated concentration of TNBS as target cells. The ordinate represents the number of PFC per spleen. The abscissa indicates the target cell used.

To determine whether the carrier molecule plays a role in this type of inhibition, Ova and BSA were tested for their inhibitory effects on anti-SRBC plaque formation. At concentrations of these proteins as high at 10^{-4} M, there was no detectable inhibition of either direct or indirect plaque formation. Thus, the carrier molecule does not appear to play a major role in this type of inhibition of plaque formation.

Anti-TNP PFC after immunization with BRBC. Since early in the immune response to a heterologous RBC, the anti-RBC plaques are inhibitable by DNP-conjugates, we attempted to determine if plaque formation could be detected after BRBC immunization by using TNP-coupled SRBC as target cells. Mice were immunized with BRBC and their spleens were assayed 3 days later for PFC on BRBC, SRBC, and SRBC coupled with 4, 20, or 40 mg TNBS/ml of packed SRBC. The target cells thus varied in their degree of TNP derivitization. It was felt that increasing the epitope density might favor plaque formation. As predicted from the inhibition data, anti-TNP-SRBC PFC were detected in BRBC-immunized mice (Fig. 2). With increasing TNP density on the SRBC, more PFC were detected. Furthermore, the number of direct anti-TNP-SRBC PFC detected in BRBC-immunized mice is greater than the number of indirect anti-TNP-SRBC PFC detected. This is

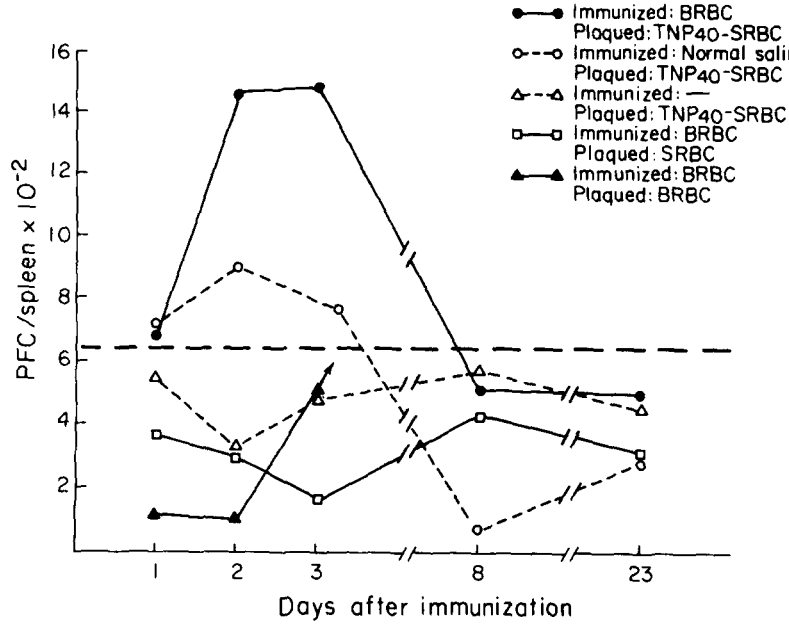

Figure 3. Incidence of plaques detected on TNP_{40}-SRBC in mice immunized with BRBC. Mice were immunized by the i.v. injection of 0.1 ml of 5% BRBC and their spleens were assayed for PFC 1, 2, 3, 8, and 23 days later by using TNP_{40}-SRBC, SRBC, and BRBC as target cells. Eight days after immunization with BRBC the number of anti-BRBC PFC exceeded the scale of this figure. Noninjected and normal saline-injected mice were also tested for their ability to produce plaques on TNP_{40}-SRBC. The ordinate represents the number of PFC/spleen and the abscissa indicates the number of days after immunization. Noninjected animals were assayed concurrently with the immunized mice.

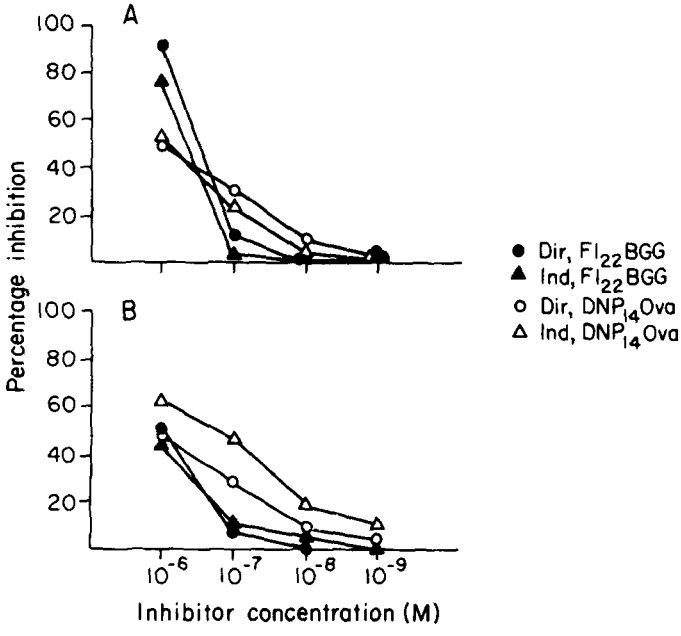

Figure 4. Inhibition of anti-TNP plaques by Fl_{22}-BGG. Mice were immunized by the i.v. injection of 100 μg DNP_{10}-KLH and their spleens were assayed for direct (Dir) and indirect (Ind) anti-TNP PFC three (panel A) and ten (panel B) days later. Fl_{22}-BGG and DNP_{14}-Ova were used as inhibitors. The ordinate represents the percentage of inhibition of the number of anti-TNP plaques and the abscissa indicates the molar concentration of inhibitor.

consistent with the findings reported above that direct plaques are more readily inhibited by DNP-carrier conjugates than are indirect plaques. It is of interest to note that there are virtually no plaques detectable on BRBC targets at a time when there are 4000 direct PFC/spleen if assayed on TNP_{40}-SRBC. It should be emphasized that when mice immunized with SRBC are assayed for PFC 2 or 3 days after immunization, comparable numbers of PFC are detected on SRBC and on high epitope density TNP-SRBC targets.[4]

The kinetics of appearance of plaques detectable on TNP_{40}-SRBC, in mice immunized with BRBC, was studied (Fig. 3). Spleens were assayed on TNP_{40}-SRBC 1, 2, 3, 8, and 23 days after injection of BRBC. The number of anti-TNP-SRBC plaques is maximal 3 days after immunization and is indistinguishable from background at 8 and 23 days. It should be noted that saline-injected and noninjected mice consistently have fewer than 800 PFC/spleen detectable on TNP_{40}-SRBC target cells. In contrast, the BRBC-injected mice consistently had at least double this background PFC level at 2 and 3 days after antigen injection. Thus, the PFC detected on TNP_{40}-SRBC 2 and 3 days after the injection of BRBC are clearly immunization related. In addition, it was confirmed that BRBC and SRBC are not cross-reactive in the classic sense. That is, no anti-SRBC PFC above background level were detected at any time after the injection of BRBC.

Inhibition of anti-TNP plaques with Fl_{22}-BGG. To determine if this type of "nonspecific" inhibition can be extended to other haptens, we immunized mice by the i.v. injection of 100 μg DNP_{10}-KLH and assayed for PFC on TNP_{20}-SRBC 3 and 10 days thereafter using DNP_{-14}Ova and Fl_{22}-BGG as inhibitors (Fig. 4). Fl_{22}-BGG was a very effective inhibitor of plaque formation on TNP_{20}-SRBC. In fact, at 3 days after antigen injection, 10^{-6} M Fl_{22}-BGG was a more effective inhibitor than 10^{-6} M DNP_{14}-Ova. Between 3 and 10 days after immunization, there was a clear decrease in the degree of inhibition by Fl_{22}-BGG but only a minimal change in inhibition by DNP_{14}-Ova. Thus, at day 10, DNP_{14}-Ova was a better inhibitor of plaque formation than Fl_{22}-BGG at all concentrations studied. The fact that only a small change in inhibition by DNP_{14}-Ova was observed is consistent with previous data that show that there is a relatively meager increase in average affinity after primary i.v. immunization without adjuvants (23). Furthermore, the use

[4] Mice immunized by the i.v. injection of SRBC averaged 540 PFC/spleen when plaqued on SRBC and 840 PFC/spleen when plaqued on TNP_{40}-SRBC 2 days after antigen injection. At 3 days after immunization with SRBC mice averaged 6380 and 8250 PFC/spleen when plaqued on SRBC and TNP_{40}-SRBC, respectively.

of a polyvalent inhibitor tends to make inhibition assays less able to discriminate between samples of different affinities (24).

DISCUSSION

We have shown that anti-SRBC plaque formation early in the course of the immune response to SRBC can be inhibited, by as much as 90%, by the presence of DNP-Ova or DNP-KLH in the agar. Similarly, we found that the anti-TNP PFC, present early after the injection of DNP-KLH, are inhibitable, by as much as 90%, by the presence of Fl-BGG in the agar. In both cases, maximum inhibition was seen 3 days after antigen injection and decreased thereafter. Since there is a well documented increase in antibody affinity with time after immunization (25), the findings suggest an inverse relationship between the degree of this type of "nonspecific" inhibition and the average affinity of the antibody secreted by the PFC. Unconjugated BGG or Ova cause no inhibition of plaque formation, and the viability of spleen cell suspensions is not altered by incubation with high concentrations the hapten-carrier conjugates used.

It was found that 3 days after immunization with SRBC, mice had the same number of PFC detectable on SRBC as on highly conjugated TNP_{40}-SRBC. At this time after antigen injection the anti-SRBC plaques are readily inhibited by DNP conjugates. This suggests that we are dealing with one antibody that is binding two different epitopes rather than with different antibodies each binding a single epitope. In the latter case one would expect to see a larger number of plaques when using the high epitope density TNP_{40}-SRBC than when using SRBC as the target cell.

The failure of the nonderivatized carrier proteins to cause inhibition of plaque formation, whereas the highly polyvalent hapten-carrier conjugates did, suggests that polyvalence may be critical in bringing about inhibition. Polyvalence might be important in two ways. a) A high epitope density would favor formation of two bonds between individual antibody and antigen molecules. This would stabilize the antigen-antibody complex and greatly increase the overall avidity of the interaction (26). b) Polyvalence might also be important in terms of the demonstrated ability of polyvalent ligands to inhibit antibody secretion by activated B cells (effector cell blockade; 27, 28).

In addition, we found that BRBC-immunized mice had PFC detectable on highly conjugated TNP-SRBC at a time after antigen injection (day 3) when no plaques could be detected on BRBC. The lack of plaques of BRBC may reflect a resistance of these cells to lysis. Thus, the BRBC reactivity of the antibody cannot be detected, whereas the TNP reactivity can lead to lysis of TNP-coupled SRBC. The fact that more PFC are detected if the TNP density on the SRBC is increased suggests that we are dealing with a heterogeneous antibody population, most likely with a low average affinity for TNP. Increasing the epitope density favors multiple-bond formation between the target RBC and individual antibody molecules (26), which would facilitate plaque formation by cells secreting low affinity antibody.

The overall data are thus consistent with the view that a large fraction of the antibody molecules present early after antigen injection is degenerate in that it can bind more than one antigenic determinant. At 3 days after antigen injection, over 90% of the antibody-secreting cells can be shown to produce antibody that is also capable of binding an unrelated antigenic determinant. It should be emphasized that the term "degenerate" is used only to indicate that the antibody molecules that are being described react with more than one ligand. The term is not meant to imply a total lack of specificity.

Sercarz and co-workers (9, 10) have shown that, at saturating concentrations of antigen and noncapping conditions, a high percentage of B cells bind more than one antigen, presumably with low affinity. It is not clear from their data whether the large number of low affinity antigen-binding cells that they detect can actually be activated by antigen to become antibody-secreting cells. Our data suggest that a high percentage of the antibody-secreting cell population present early after immunization is also degenerate. This implies that the degenerate low affinity antigen-binding cells detected by DeLuca *et al.* (9, 10) can indeed be activated by antigen. We have previously reported that very large amounts of low affinity antibody is secreted in the normal immune response (29–31). These findings are also consistent with the concept that low affinity antigen-binding cells can be activated by antigen to become antibody-secreting cells.

The phenomenon of one antibody molecule binding more than one antigenic determinant has been elegantly demonstrated by Richards and co-workers (11–14). Cameron and Erlanger (32) have also obtained evidence that apparently homogeneous antibodies can bind multiple unrelated antigens. Richards and co-workers (11–14) screened extensively for cross-reactivity between structurally unrelated haptens and detected five cross-reactive pairs after testing 144 haptens against secondary anti-DNP antiserum. It is interesting that they were looking late in the immune response when the affinity of the antibody for the immunizing antigen is very high (25). One would expect that degeneracy should be more readily demonstrable with early, low affinity, primary-response antibody. By making the detection system very sensitive (high epitope density), we were able to demonstrate degenerate antibody-secreting cells, in high incidence, early in the immune response.

There are certain superficial similarities between this type of B cell activation by "antigen" and polyclonal B cell activation by "mitogen." Several aspects of the data are compatible with the criteria for polyclonal B cell activation formulated by Möllers group and Gronowicz and Coutinho (33, 34). For example, after immunization with BRBC: a) PFC are detected with high epitope density TNP_{40}-SRBC as target cells; b) the degenerate response consists mainly of IgM antibodies; and c) the degenerate response peaks 3 days after immunization. On the other hand, it would be hard to interpret the very high percentage of apparently degenerate PFC as due to polyclonal B cell activation. Another reason why we doubt that the apparent degeneracy is simply a reflection of polyclonal B cell activation is the decrease in degeneracy with time after immunization. This suggests that as high affinity cells are selected, the degree of degeneracy decreases. It would seem logical to postulate that the antigen receptors on the antigen-binding cells are degenerate and that these cells can be activated by interaction with antigen early in the immune response. As selection for cells producing high affinity antibodies takes place (25), the incidence of detectable degenerate PFC decreases.

It appears that low affinity antibody shows a marked degree of degeneracy if conditions favor its detection. One would predict that antibody that is of high affinity for the immunizing antigen would exhibit less degeneracy. That is, it is unlikely that antibody molecules that bind one antigenic determinant with high affinity would also bind a second randomly selected antigenic determinant. As the population of high affinity antibody producing cells is selectively expanded during the course of the immune response, one would expect the degree of degen-

eracy of PFC to decrease, as was observed. The data thus suggest that, early in the immune response, many degenerate low affinity antibody-producing cells are activated by antigen. With time, the cell population shifts so as to consist of less degenerate, high affinity antibody-secreting cells. Theoretically, all antibody molecules are probably degenerate; however, this degeneracy is not readily detected when a high affinity subpopulation is examined. Presumably, if a large panel of ligands were tested, degeneracy of these "high affinity" antibodies could also be demonstrated.

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