

PRESENCE OF PLASMA CELLS BINDING AUTOLOGOUS  
ANTIBODY DURING AN IMMUNE RESPONSE\*

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The possibility that the immune system might be regulated, at least in part, by idiotype-anti-idiotype interactions was first proposed by Jerne (1). Support for this theory has come from the demonstration of spontaneously appearing antibodies and/or plaque-forming cells specific for the T15 or E109 idiotypic determinants on immunoglobulin molecules produced in inbred mice immunized with phosphorylcholine or levan, respectively (2-4); a similar anti-idiotypic response was observed in rats repeatedly immunized with membrane alloantigens (5). Other experiments have demonstrated that anti-idiotypic antibodies can be induced in either autologous (6) or heterologous systems, and that the immune response can be altered by the administration of these antibodies (7, 8).

To provide further support for the theory of idiotype-anti-idiotype regulation, one should be able to show the presence of cells producing antibodies to an individual's own idiotypic determinants during an immune response. In this communication we demonstrate by immunofluorescence techniques that this is indeed the case; plasma cells capable of binding autologous antibody can be detected in outbred animals.

**Materials and Methods**

*Animals and Immunizations.* Female New Zealand white rabbits, weighing 3-4 kg, were immunized by daily i.v. injections on alternate weeks of 5 mg human serum albumin (HSA) (9). Crystalline HSA (Sigma Chemical Co., St. Louis, Mo.) was further purified by Pevikon block electrophoresis and passage over Affigel-Blue (Bio-Rad Laboratories, Richmond, Calif.), which specifically binds albumins. Human lactoferrin (LF) was prepared as previously described (10); antibody to this antigen was induced by i.v. immunization as for HSA.

*Purification and Fluorochrome Labeling of Immunoglobulins.* Blood was taken when the rabbits were killed, 7-35 d after the final injection of antigen. The preparation of serum IgG and F(ab')<sub>2</sub> fragments, and subsequent labeling with tetramethylrhodamine isothiocyanate (TRITC) have been described (11). F(ab')<sub>2</sub> fragments, separated on a Sephadex G-200 column, had a sedimentation constant of 5S. Fab' fragments were obtained by reduction of conjugated F(ab')<sub>2</sub> with 0.01 M 2-mercaptoethanol for 8 h, followed by overnight alkylation with 0.01 M iodoacetamide, and subsequent purification on a column of Sephadex G-200. HSA was conjugated to fluorescein isothiocyanate (FITC) or TRITC in a manner similar to that of the antibody preparations.

Fluorochrome-conjugated antisera specific for heavy chains of rabbit IgM, IgG, and IgA were used to determine the class of Ig in anti-idiotype-producing cells. These reagents were rendered specific for  $\alpha$ - and  $\mu$ -chains and the Fc fragment of IgG by absorption through a column of polyclonal IgG or F(ab')<sub>2</sub> coupled to cyanogen bromide-activated Sepharose.

*Tissues.* Spleen, bone marrow, popliteal or mesenteric lymph nodes, and intestine were

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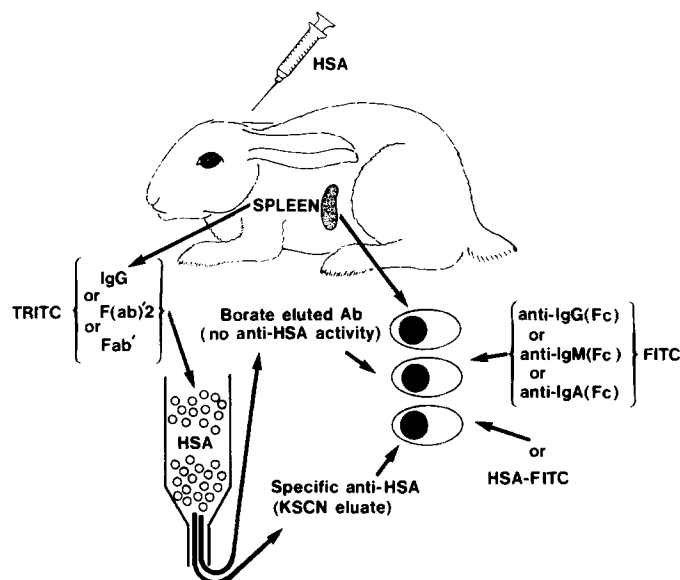


FIG. 1. Experimental design. Animals were immunized i.v. for various periods of time (Table I). When an experiment was terminated, rabbits were killed by cardiac exsanguination and either TRITC-labeled IgG, F(ab)<sub>2</sub>, or Fab fragments were prepared. The labeled antibodies or fragments were purified by affinity chromatography on an antigen column and used to stain preparations of autologous splenic cells, taken at time of killing. The cells were costained with FITC-labeled anti-rabbit Ig reagents specific for the Fc portion of IgG, IgM, or IgA. Cells binding antigen were counted after staining of another slide with TRITC-labeled HSA and costaining with FITC-anti-Ig.

either quick-frozen in liquid nitrogen or processed for cytocentrifuge slides (prepared in phosphate-buffered saline with 1% bovine gamma globulin or 0.1% gelatin). Tissue or cell preparations were processed for the detection of antigen-binding cells (12). Specimens were examined in a fluorescence microscope equipped with a vertical illuminator and a set of filters for narrow band excitation (Orthoplan, Leitz, West Germany).

**Isolation of Specific Anti-HSA.** Conjugated antibody was passed through a column of HSA-Sepharose in borate buffer until no anti-HSA activity could be detected by passive hemagglutination (PHA), using HSA-coated sheep erythrocytes. Specifically bound antibody was eluted with 3 M potassium thiocyanate and subsequently passed through a column of anti-HSA-Sepharose or Affigel-Blue to remove any antigen or antigen-antibody complexes that coeluted with specific antibody.

### Results and Discussion

The procedures used for the detection of cells that bind: (a) HSA-specific IgG (idiotype), isolated from auto- or alloantisera; (b) HSA (antigen); or (c) FITC-labeled reagents to rabbit immunoglobulins are outlined in Fig. 1. This method has the advantage of permitting us to detect the presence of cells producing anti-idiotypic antibodies (Fig. 2) to the animal's own specific antibody, and of avoiding the use of heterologous or homologous systems. The antibodies that we refer to as idiotypic represent a heterogeneous population of Ig molecules with anti-HSA activity, as evidenced by isoelectric focusing analyses.

In Table I, data are summarized that show the percentage of Ig-positive cells that bind antigen or idiotype antibody (anti-idiotypic-producing cells) as related to the length of the immunization period. It is apparent that there is a great variability

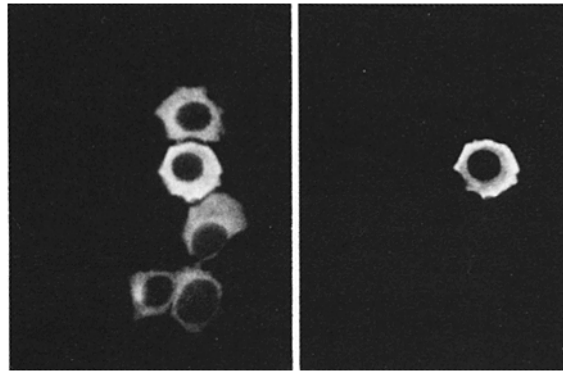


FIG. 2. Cytocentrifuge slide of spleen cells from rabbit No. 5 stained with FITC-labeled goat anti-IgG (Fc) (left) and autologous TRITC-labeled  $F(ab')_2$ -specific anti-HSA (right).

TABLE I  
*Idiotypic- and HSA-binding Splenic Plasma Cells of Rabbits Immunized with HSA or LF*

Anti-HSA or anti-LF from rabbit No.	Length of immunization	Percentage of spleen cells in rabbit binding idiotype (anti-HSA or anti-LF)*							
		1	2	3	4	5	6	7	8§
	<i>d</i>	%							
1	16	0 (0)	0.2	0.1	0	36.1	ND¶	0.7	0
2	36	0	6.5 (0.5)	0.7	1.0	27.2	5.6	1.3	0
3	64	0	1.4	0.7 (0.5)	3.2	41.7	ND	2.7	0
4	80	0	0.3	0.5	4.7 (3.0)	41.8	2.5	1.3	0
5	158	0	0.4	0.5	3.0	44 (2.6)	18.2	2.0	0
6	354	0	0.2	0.4	0.7	36.5	20.2	1.3	0
7	542	0	0.3	0.7	3.7	46.9	ND	2.7 (0.7)	0
8‡	62	0	0	0	0	0	0	0	8.3
9§	57	0	0	0	0	0	0	0	7.1
% HSA-binding cells		0.1	0.6	0.1	8.1	46.9	3.8	2.1	0

\* Expressed as percentage of Ig-positive cells. Cytocentrifuge slides were stained with Fc-specific FITC-labeled goat anti-rabbit Ig reagents and subsequently with TRITC-labeled HSA, or anti-HSA ( $F[ab']_2$  fragment), or anti-LF. Approximately 300–1,000 Ig-positive cells were counted per slide. Identical numbers of idiotype-binding cells were observed when slides were stained in the absence of anti-Ig reagents.

‡ Immunized with LF.

§ Antiserum previously prepared in this laboratory from a rabbit immunized with LF in complete Freund's adjuvant (10).

|| Italicized numbers represent binding of specific anti-HSA to spleen cells of autologous origin. Values in parentheses are those obtained when the fraction of labeled antibody that had no HSA-binding ability (borate eluate) was applied on autologous cells.

¶ ND, not determined.

among individual animals, and that there is a high degree of idiotypic cross-reactivity among the anti-HSA preparations from different rabbits. However, it should be noted that the percentage of positive cells was highest when autologous antibody was used for staining. The autologous values were sometimes equaled when a labeled preparation from another animal was used, but cross-staining experiments generally yielded numbers lower than those obtained when the animal's own antibody was used to stain its own cells. Idiotypic cross-reactivity was also observed when a previously prepared rabbit anti-LF reagent was used to stain the cells of an LF-immunized rabbit. No cells that bound autologous anti-HSA antibody were detected in a rabbit immunized for only 16 d, although HSA-binding cells were present, and labeled antibody from this animal did stain the cells from other HSA-immunized animals.

Only a small proportion of cells were stained with that portion of labeled IgG that did not adhere to the HSA column. These results suggest that most of the observed staining could be ascribed to that fraction of antibody that contained anti-HSA activity. However, a small proportion of idiotype-bearing molecules that did not bind HSA were present in these animals; this phenomenon has been observed by others (7). Identical results were obtained when either TRITC-labeled IgG of idiotype, F(ab')<sub>2</sub>, or Fab' were used to detect anti-idiotype-producing cells. These findings suggest that anti-idiotype-producing cells bind anti-HSA through the Fab' fragment. By using TRITC-labeled F(ab')<sub>2</sub> or Fab' of idiotype and FITC-labeled goat anti-rabbit isotype reagents, we could determine the antibody class in cells that bound idiotype. With the exception of rabbit No. 2, ~30% of whose anti-idiotype cells were of the IgM class, the majority (>95%) of anti-idiotype-positive cells were of the IgG class. Cells binding idiotype were also observed in bone marrow, rarely in popliteal and mesenteric lymph nodes, but not in intestinal tissues.

Several experiments were performed to confirm the specificity of the observed reactions. Antigen specificity was demonstrated by the absence of staining of cells from HSA-immunized rabbits with labeled preimmune IgG or rabbit TRITC-labeled antibodies to human LF, J chain, IgA, IgM, IgG, bacteriophage  $\phi$ X174, and *Streptococcus mutans*. On the other hand, spleen cells from a rabbit immunized with human LF bound both autologous TRITC-labeled anti-LF as well as another anti-LF previously prepared in our laboratory, but none of the anti-HSA reagents. Likewise, spleens of rabbits immunized with bacteriophage  $\phi$ X174, *S. mutans*, and J chain were stained in low numbers only with autologous antibody (S. Jackson, R. Kulhavy, and J. Mestecky. Manuscript in preparation.).

The antigen specificity of the staining, as well as the fact that preabsorption of the conjugates with aggregated rabbit IgG did not affect the numbers of positive cells, indicated that our results were not influenced by the presence of a serum rheumatoid factor (RF). The presence of intracellular RF was excluded by the finding that no differences were noted in total numbers of cells binding idiotype when TRITC-labeled IgG, F(ab')<sub>2</sub>, or Fab' fragments were used, or if slides were incubated with normal rabbit IgG before staining.

The possibility that staining might be due to the presence of in vivo-formed circulating antigen-antibody complexes, which would bind through accessible determinants of HSA, was excluded by two experiments. First, gel filtration of conjugated antibody on Sephadex G-200 in glycine-HCl buffer, pH 2.4, which would be expected to dissociate any such complexes, had no effect on staining. In the second experiment, the conjugate was passed through Affigel-Blue or anti-HSA columns, thus removing any staining mediated through the antigen. Neither of these treatments affected the numbers of positive cells counted.

To demonstrate that the observed idiotype- or antigen-binding activity resided within cells, cytocentrifuge slides were treated with 10 M urea (pH adjusted to 2.5 with 4 M acetic acid) or reduced (0.2 M 2-mercaptoethanol) and subsequently alkylated (0.2 M iodoacetamide) before staining with TRITC-idiotype or FITC-goat anti-rabbit Ig. Although either of these treatments abolished staining with TRITC-labeled idiotype or HSA, staining with FITC anti-Ig was not affected. These results indicate that some antigenic determinants of intracellular Ig apparently remain present despite this treatment, whereas the ability to bind idiotype or HSA within the

cells was abolished. These findings also imply that staining was not a result of the presence of intracellular antigen, because it is unlikely that these treatments would remove or completely destroy antigenic determinants of HSA but not immunoglobulin. Additionally, if antigen were present, one would expect to observe the same percentage of positive cells regardless of the origin of anti-HSA. The data given in Table I show that this is not the case.

These data indicate that an autologous anti-idiotypic response is induced as a consequence of antigenic stimulation; however, no staining was observed when normal rabbit IgG was applied on autologous spleen cells or when the experiment was performed on a rabbit immunized for only 16 d. This suggests that the anti-idiotypic response may not be detectable under normal conditions. Tasiaux et al. (13), in experiments similar to ours, demonstrated surface receptors for idiotypic determinants on peripheral lymphocytes of rabbits immunized with tobacco mosaic virus, disclosed by staining with autogeneic and allogeneic antisera. Cross-reactive idiotypes on antibody molecules with similar binding abilities, isolated from different individuals of the same species, were observed first in humans (14) and have also been demonstrated in other animals, particularly in rabbits and mice (8).

Unexpectedly high numbers of antigen and idiotypic-binding cells were found in cytocentrifuge slides as well as tissue sections of spleen from rabbit No. 5. With the exception of bone marrow, where idiotypic-binding cells were also present, other tissues, such as lymph nodes, lacked these cells. At present, we can offer no explanation concerning the almost exclusive distribution and high percentage of idiotypic- and antigen-binding cells in the spleen of this rabbit.

The number of cells binding idiotypic is surprisingly high when numbers of antigen-binding cells are considered. However, attempts to detect circulating anti-idiotypic by reverse PHA were unsuccessful. Therefore, the fate of anti-idiotypic antibody and its *in vivo* function are at present unknown.

### Summary

Spleen and other lymphoid tissues of rabbits immunized with human serum albumin (HSA) and human lactoferrin (LF) were examined for the presence of cells forming anti-idiotypic antibodies. To detect these cells, IgG, F(ab')<sub>2</sub>, or Fab' of specific antibodies were isolated, fluorochrome-tagged with tetramethylrhodamine isothiocyanate, and used as an idiotypic marker to detect splenic plasma cells that are producing anti-idiotypic antibody. By this procedure, we were able to demonstrate anti-idiotypic cells in surprisingly high numbers. For example, in six rabbits immunized with HSA for periods ranging from 36 to 542 d, the percentage of Ig-positive cells that stained with autologous idiotypic ranged from 0.7 to 44; furthermore, cross-reactivity was observed among seven different anti-HSA preparations and two anti-LF antisera. The isotype of anti-idiotypic cells, determined by costaining with fluorescein isothiocyanate-labeled goat Fc-specific anti-rabbit Ig, was shown to be predominantly IgG. These findings provide evidence of the presence of plasma cells producing antibody to autologous idiotypic during a vigorous immune response.

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### References

1. Jerne, N. K. 1974. Towards a network theory of the immune system. *Ann. Immunol. (Paris)*. **125C**:373.
2. Kluskens, L., and H. Köhler. 1974. Regulation of immune response by autogenous antibody against receptor. *Proc. Natl. Acad. Sci. U. S. A.* **71**:5083.
3. Cosenza, H. 1976. Detection of anti-idiotypic reactive cells in the response to phosphorylcholine. *Eur. J. Immunol.* **6**:114.
4. Bona, C., R. Lieberman, C. C. Chien, J. Mond, S. House, I. Green, and W. E. Paul. 1978. Immune response to levan. I. Kinetics and ontogeny of anti-levan and anti-inulin antibody response and of expression of cross-reactive idiotype. *J. Immunol.* **120**:1436.
5. McKearn, T. J., F. P. Stuart, and F. W. Fitch. 1974. Anti-idiotypic antibody in rat transplantation immunity. I. Production of anti-idiotypic antibody in animals repeatedly immunized with alloantigens. *J. Immunol.* **113**:1876.
6. Rodkey, L. S. 1974. Studies of idiotypic antibodies. Production and characterization of autoantiidiotypic antisera. *J. Exp. Med.* **139**:712.
7. Eichmann, K. 1978. Expression and function of idiotypes on lymphocytes. *Adv. Immunol.* **26**:195.
8. Capra, J. D., and J. M. Kehoe. 1975. Hypervariable regions, idiotype, and the antibody-combining site. *Adv. Immunol.* **20**:1.
9. Chase, M. W. 1967. Immunization Procedures. I. Immunization of mammals other than man. In *Methods in Immunology and Immunochemistry*. Volume 1. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. 209.
10. Cole, M. F., R. R. Arnold, J. Mestecky, S. Prince, R. Kulhavy, and J. R. McGhee. 1976. Studies with human lactoferrin and *Streptococcus mutans*. In *Microbial Aspects of Dental Caries*. H. M. Stiles, W. J. Loesche, and T. C. O'Brien, editors. Information Retrieval, Inc., Washington, D. C. 359.
11. Winchester, R. J. 1976. Techniques of surface immunofluorescence applied to the analysis of the lymphocyte. In *In Vitro Methods in Cell-Mediated and Tumor Immunity*. B. R. Bloom and J. R. David, editors. Academic Press, Inc., New York. 171.
12. Hijmans, W., H. R. E. Schuit, T. Yamashita, and I. Schechter. 1972. An immunofluorescence study on the specificity of antibodies synthesized in separate cells after the administration of an immunogen with double specificity. *Eur. J. Immunol.* **2**:1.
13. Tasiaux, N., R. Leuwenkroon, C. Bruyns, and J. Urbain. 1978. Possible occurrence and meaning of lymphocytes bearing autoanti-idiotypic receptors during the immune response. *Eur. J. Immunol.* **8**:464.
14. Williams, R. C., H. G. Kunkel, and J. D. Capra. 1968. Antigenic specificities related to the cold agglutinin activity of gamma M globulins. *Science (Wash. D. C.)*. **161**:379.