

CROSS-TOLERANCE BETWEEN SEROLOGICALLY NON-CROSS-REACTING FORMS OF EGG WHITE LYSOZYME*

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A functional division within the immune response has become generally accepted in recent years. Functions associated with cell-mediated immunity are mainly dependent upon the thymus-influenced (T) lymphocyte (1, 2), whereas antibody production is generally dependent upon the non-thymus-influenced (B) lymphocyte (3). However, there is considerable evidence that, for at least some antigens, T cells also participate in the antibody response (4, 5). A considerable body of knowledge regarding the nature and function of the two cell types has accumulated, but little is known concerning their relative specificities. Several reports have dealt with cellular cross-reactions between antigens which exhibit little or no humoral cross-reactivity (6-8), a phenomenon which suggests differences in the specificities of the two cell types. A report from this laboratory (9) demonstrated a similar phenomenon with egg white lysozyme and its reduced and carboxymethylated derivative, CM-lysozyme. These two forms of lysozyme, which are well documented to be non-cross-reactive at the humoral level (9-11) were shown to cross-react with respect to several parameters of the immune response, namely delayed skin reactivity, migration-inhibitory factor production, stimulation of splenic lymphocytes, and cyclophosphamide-induced tolerance. The experiments presented herein demonstrate that this cross-reactivity extends to neonatally and adult-induced tolerance.

Materials and Methods

Antigens.—Hen egg white lysozyme (Muramidase), 3X crystallized and salt free, was purchased from Miles Laboratories, Inc., Elkhart, Ind. Reduction and alkylation of lysozyme was carried out according to the method of Canfield and Anfinsen (12).

Animals.—Mice of the BAB/14 strain were used throughout.

Tolerance Induction.—Neonatal mice received one of the following subcutaneous treatments with lysozyme in saline, commencing on the 1st day of life: 100 μ g on days 1-20; 500 μ g on day 1; 1 mg on days 1-20; 5 mg on day 1; 5 mg on days 1-2; and 5 mg on days 1-4. All neonatally treated mice received primary challenge at 6 wk of age. Tolerance in adult animals (6-8-wk old) was induced by intraperitoneal administration of 50 mg of lysozyme in 0.5 ml of saline; primary challenge was given 10 days later.

Immunization.—All animals were challenged with 100 μ g of either lysozyme or CM-

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lysozyme in 0.2 ml of complete Freund's adjuvant (*Mycobacterium tuberculosis* H37Ra; Difco Labs., Inc., Detroit, Mich.) administered to all four footpads and intraperitoneally. This was followed 2 wk later with 100 μ g of antigen and 100 μ g of Benadryl (Parke, Davis & Company, Detroit, Mich.) in 0.1 ml of saline administered intraperitoneally. All mice were bled from the tail vein 2 wk after the secondary challenge, the time previously established to be the peak of the normal response elicited by this immunization schedule.

Antibody Assay.—Individual sera were diluted 1/10, 1/100, and 1/1000 in borate (0.05 M)-buffered saline (BBS), pH 8.0. The two higher dilutions were supplemented with normal mouse serum to equalize the protein concentration. A mixture of 0.1 ml of each serum dilution and 0.1 ml of a 1.0 μ g/ml solution of antigen-¹²⁵I in 0.1 M NaHCO₃ was incubated for 30 min at room temperature and precipitated by an excess of hyperimmune rabbit antiserum to mouse globulins. The tubes were counted in a Nuclear-Chicago automatic gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.) before centrifugation and after centrifugation and two washes with BBS. The per cent of antigen bound was calculated according to the formula:

$$\frac{X - (Y - X)K}{Y}$$

where X represents counts precipitated, Y represents total counts added,

and K represents that fraction of the antigen precipitated by normal mouse serum under comparable conditions (i.e., background).

The antigen-binding capacity (ABC-33) was determined by plotting the per cent antigen bound on a linear scale vs. serum dilution on a log scale. From the resulting line, that dilution of antiserum which bound 33% (0.033 μ g) of the antigen was determined, and a direct conversion was made to the micrograms of antigen bound by 1 ml of undiluted antiserum. In some cases the binding was too low to assess the 33% point even by extrapolation. In these cases the ABC was calculated directly from the 1/10 dilution.

Iodination.—Iodination of lysozyme and CM-lysozyme was according to the method of McConahey and Dixon (13). Unbound iodine was removed by passing the reaction mixtures through 1 \times 40 cm columns of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with 0.1 M NaHCO₃.

RESULTS

The responses to lysozyme or to CM-lysozyme of animals which had undergone various tolerization regimens with lysozyme are shown in Table I. When compared with the response of normal animals, all treatments except one (100 μ g for 20 days) resulted in either severe suppression or outright abolition of the antibody response to lysozyme. Moreover, animals which had undergone identical treatments with lysozyme were also tolerant to CM-lysozyme. In contrast (data not shown), mice given 20 mg of bovine serum albumin over the first 4 days of life responded normally to both lysozyme and to CM-lysozyme, and lysozyme-tolerant animals (as in group D, Table I) responded to a non-related antigen, tobacco mosaic virus protein, in a normal manner.

DISCUSSION

There are several reports which indicate that immunological cross-reactivity at the level of delayed hypersensitivity may exist between antigens which are not necessarily cross-reactive at the humoral level (6–8). In a previous report from this laboratory (9), evidence was presented for immunological cross-reaction between lysozyme and CM-lysozyme as assessed by several measures of cellular immunity, although the two forms are serologically non-cross-reactive.

tive. The present communication offers evidence for cross-reaction with respect to still another immunological parameter, namely, the existence of cross-tolerance between these two antigens.¹

Delayed hypersensitivity is now generally accepted as being a function of the thymus-influenced (T) lymphocyte (1, 2). The lack of humoral (B lymphocyte) cross-reactivity in the present system suggests that T cells are responsible for the cross-reactions observed with respect to the other parameters which we have reported (9). Consequently, if tolerance is operative at the level of both T and B cells, it would be expected that tolerization to lysozyme would also render the animals tolerant to CM-lysozyme by virtue of their cross-reactivity at the T cell level. The data presented in this communication support

TABLE I
Cross-Tolerance between Lysozyme and CM-Lysozyme

| Group | Treatment* | Response to lysozyme | | Response to CM-lysozyme | |
|-------|----------------|----------------------|---------------|-------------------------|-------------|
| | | Responders‡ | Titer | Responders‡ | Titer |
| A | None | 11/11 | (16.4 ± 5.3) | 9/9 | (4.3 ± 2.4) |
| B | 100 µg/20 days | 4/4 | (6.4 ± 2.6) | N.D.§ | — |
| C | 500 µg/1 day | 2/6 | (2.4 ± 2.1) | 0/4 | 0 |
| D | 1 mg/20 days | 3/10 | (0.5 ± 0.3) | 0/11 | 0 |
| E | 5 mg/1 day | 2/9 | (0.85 ± 0.5) | 0/3 | 0 |
| F | 5 mg/2 days | 4/13 | (0.36 ± 0.43) | 0/6 | 0 |
| G | 5 mg/4 days | 2/6 | (0.12 ± 0.01) | 0/6 | 0 |
| H | 50 mg/1 day | 6/6 | (1.7 ± 1.5) | 1/6 | (1.1) |

* Amount of lysozyme given per day/No. of days administered, commencing on 1st day of life.

‡ No. of responders/No. challenged. Titer is ABC-33 (mean ± SD) of responders only.

§ Not done.

|| Adult-induced tolerance, 6-8-wk old animals given 50 mg of lysozyme.

this hypothesis. Furthermore, this view is consistent with the finding that mice rendered tolerant by the lower tolerogen doses employed here produced antibodies to lysozyme when challenged with a conjugate of lysozyme and a nonrelated protein,² suggesting that only the helper (T) cell activity has been eliminated from the tolerant animals. T cells are thus capable of recognizing similarities between two antigens which are totally segregated at the B cell level. Moreover, in view of the extent of the cross-tolerance, the tolerogen (lysozyme) must have affected a majority, if not the total, of the T cells capable of recognizing CM-lysozyme.³

¹ Some years ago Austin and Nossal observed a cross-tolerant state between several serologically non-cross-reacting flagella from *Salmonella sp.* (14). The cross-reactivity of these flagella with respect to other immunological parameters was not investigated.

² R. Scibienski, S. Fong, M. Harris, and E. Benjamini. Manuscript in preparation.

³ It is unlikely that the CM-lysozyme tolerance observed is the result of contamination of the tolerogen with denatured forms. Inhibition studies revealed the maximal degree of denaturation of the tolerogen to be less than 0.1% (R. Scibienski, unpublished results), whereas 10% of a tolerance-inducing dose had only a minimal suppressive effect on the response to lysozyme (group B vs. group D, Table I).

T cell cross-reactivity in the absence of B cell cross-reactivity implies that there are basic differences either in the antigenic specificity of the two cell types or in their activation requirements. Consistent with one or both of these ideas are the findings that T cell-associated genetic control of immune responsiveness is not linked to any of the known immunoglobulin allotypes in the mouse (15), the paucity (16), if not the absence (17, 18), of immunoglobulin on the surface of T cells, and the exquisite sensitivity of T cells to antigen as compared with B cells (19). The basis of these differences is not known, but the present results suggest that T cells may be less dependent on conformational features than are B cells.

REFERENCES

1. Miller, J. F. A. P., A. H. E. Marshall, and R. J. White. 1962. The immunological significance of the thymus. *Adv. Immunol.* **2**:111.
2. Good, R. A., A. P. Dalmasso, C. Martinez, O. K. Archer, J. C. Pierce, and B. W. Papermaster. 1962. The role of the thymus in development of immunologic capacity in rabbits and mice. *J. Exp. Med.* **116**:773.
3. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.
4. Miller, J. F. A. P., A. Basten, J. Sprent, and C. Cheers. 1971. Interactions between lymphocytes in the immune response. *Cell. Immunol.* **2**:469.
5. Claman, H. N., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells. A model for the two cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
6. Gell, P. G. H., and B. Benacerraf. 1959. Studies on hypersensitivity. II. Delayed hypersensitivity to denatured proteins in guinea pigs. *Immunology.* **2**:64.
7. Parkhouse, R. M. E., and R. W. Dutton. 1967. The effect of physical and chemical modifications of antigens in the secondary response in vitro. *Immunochemistry.* **4**:431.
8. Parish, C. R. 1971. Immune response to chemically modified flagellin. II. Evidence for a fundamental relationship between humoral and cell-mediated immunity. *J. Exp. Med.* **134**:21.
9. Thompson, K., M. Harris, E. Benjamini, G. F. Mitchell, and M. Noble. 1972. Cellular and humoral immunity: a distinction in antigenic recognition. *Nat. New Biol.* **238**:20.
10. Gerwing, J., and K. Thompson. 1968. Studies on the antigenic recognition of egg white lysozyme. I. Isolation and characteristics of a tryptic peptide from reduced and alkylated lysozyme exhibiting haptenic activity. *Biochemistry.* **7**:3888.
11. Young, J. D., and C. Y. Leung. 1970. Immunochemical studies on lysozyme and CM-lysozyme. *Biochemistry.* **9**:2755.
12. Canfield, R. E., and C. B. Anfinsen. 1963. Chromatography of pepsin and chymotrypsin digests of egg white lysozyme on phosphocellulose. *J. Biol. Chem.* **238**:2684.

13. McConahey, P. J., and F. J. Dixon. 1966. A method for trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.
14. Austin, C. M., and G. J. V. Nossal. 1966. Mechanism of induction of immunological tolerance. III. Cross-tolerance amongst flagellar antigens. *Aust. J. Exp. Biol. Med. Sci.* **44**:341.
15. McDevitt, H. O., K. B. Bechtol, F. C. Grumet, G. F. Mitchell, and T. G. Wegman. 1971. Genetic control of the immune response to branched synthetic polypeptide antigens in inbred mice. *In Progress in Immunology, Proceedings of the First International Congress of Immunology*. Bernard Amos, editor. Academic Press, New York.
16. Hammerling, U., and K. Rajewsky. 1971. Evidence for surface-associated immunoglobulin on T and B lymphocytes. *Eur. J. Immunol.* **1**:447.
17. Unanue, E. R., H. M. Grey, E. Rabellino, R. Campbell, and S. Schmidtke. 1971. Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the main source of lymphocytes with detectable surface-bound immunoglobulin. *J. Exp. Med.* **133**:1188.
18. Takahashi, T., L. J. Old, K. R. McIntire, and E. A. Boyse. 1971. Immunoglobulin and other surface antigens of cells of the immune system. *J. Exp. Med.* **134**:815.
19. Mitchison, N. A. 1971. The relative ability of T and B lymphocytes to see protein antigen. *In Cell Interactions and Receptor Antibodies in Immune Responses, Proceedings of the Third Sigrid Juselius Symposium*. O. Mäkelä, A. Cross, and T. U. Kosunen, editors. Academic Press, New York.