SUPPRESSION OF MEMORY BY PASSIVE IMMUNIZATION LATE IN THE PRIMARY RESPONSE*

BY MICHAEL A. AXELRAD, M.D.

(From the Department of Pathology, Queen's University, Ontario, and the Kingston General Hospital, Kingston, Ontario, Canada)

(Received for publication 4 November 1970)

The antibody-forming cell response in the spleens of mice given intravenous sheep erythrocytes (SRBC) can be monitored by use of the hemolytic plaque-forming cell assay (1, 2) which, if supplemented by the use of anti-mouse IgG "developing serum," (3) enables the demonstration of IgG plaque-forming cells (indirect PFC) in addition to IgM plaque-forming cells (direct PFC). In such a system the primary response to $1.5 \times 10^8$ SRBC is characterized by an initial exponential rise of direct PFC trailed at an interval of about 2 days by a similarly swift generation of indirect PFC; the entire detected response is aborted if a small amount of late-immune anti-SRBC serum is given a few hours before the antigen. The response to a second injection of SRBC, given a few weeks after the primary, differs in several respects including an acquired insusceptibility to inhibition by anti-SRBC serum. This readily measurable insusceptibility was chosen as a criterion for the presence of immunological memory. It was found that late-immune anti-SRBC serum given too late to affect the peak primary direct PFC response to SRBC remained highly potent in preventing both the usual subsequent indirect PFC response and the establishment of memory. The procedure of such delayed passive immunization provided a simple tool for dissecting a sequence of changes after primary immunization.

Materials and Methods

Animals.—Swiss white mice (Charles River Breeding Laboratories, Wilmington, Mass.), 7-14 wk old and of both sexes, but in groups matched for age and sex within each experiment, were used throughout. Anti-mouse IgG was raised in male NZW rabbits (Hazelbrook Beagles, London, Ontario, Canada), and complement was obtained from mature female guinea pigs (Canadian Breeding Farm & Laboratories Ltd., St. Constant, Quebec, Canada).

Antigens.—Sterile washed sheep erythrocytes were always obtained from the same male gelding, collected in Alsever's, and used within 1 wk of collection. Mouse IgG was separated

* This study was supported by grant MA-3153 from the Medical Research Council of Canada.

Abbreviations used in this paper: direct PFC, IgM plaque-forming cells; indirect PFC, IgG plaque-forming cells; SRBC, sheep erythrocytes.
in an immunoelectrophoretically pure form from ascitic fluid induced by Ehrlich ascites tumor inoculation. Initial precipitation with 50% ammonium sulphate was followed by dialysis against 0.01 M phosphate buffer at pH 9 and subsequent diethylaminoethyl-cellulose column chromatography with elution at pH 7.0 in 0.01 M phosphate.

Sera.—Late-immune mouse anti-SRBC serum was obtained by cardiac exsanguination of a pool of 200 mice given $3 \times 10^8$ SRBC intraperitoneally 8 and 3 wk before sacrifice. The pooled serum was inactivated at 56°C for 30 min and stored frozen in small portions; it gave an SRBC agglutinin titer of 1/640 which was not reduced by incubation in 0.1 M 2-mercaptoethanol at 37°C for 30 min. Rabbit anti-mouse IgG was collected 10 wk after commencement of a course of two injections of purified mouse IgG in a dose of 500 µg/kg, the first being given with Freund complete adjuvant. Specificity was tested by immunoelectrophoresis. The serum was inactivated at 56°C for 30 min, absorbed with SRBC, lyophilized, and stored at --20°C as small samples in sealed, evacuated ampules. It was used after reconstitution at a dilution of 1/20. Freshly obtained guinea pig serum was lyophilized and stored at --20°C for use as complement at a dilution of 1/8 after reconstitution.

Immunization.—Each active immunization with SRBC took the form of $1.5 \times 10^8$ SRBC intravenously. Passive immunization comprised 0.4 ml of late-immune anti-SRBC serum given intraperitoneally.

Plaque-Forming Cell Assay.—This followed the method of Jerne et al. (2) with additional modifications for demonstration of indirect plaque-forming cells as described by Wortis et al. (4).

RESULTS

Passive Immunization before Antigen Administration.—Preliminary experiments established that the pool of late-immune mouse anti-SRBC serum employed could effectively inhibit a PFC response to sheep erythrocytes. Given 24 hr before primary immunization, this antiserum abolished a subsequent 4 day response; similar administration before secondary immunization had little effect on the PFC response which followed (Table I). The markedly different effects of such an antiserum on primary and secondary immune responses appeared to clearly distinguish “immune” from “nonimmune” animals, and was taken as a measure of the presence of immunological memory.

Passive Immunization after Antigen.—If administration of the antiserum was delayed for 3 days after giving antigen, no effect on the peak primary 4 day direct PFC response was observed. In view of data suggesting that memory has not yet been established at this time (5) the possibility that such delayed passive immunization may yet affect memory was assessed, by testing the secondary response of mice which had received anti-SRBC serum 3 days after primary immunization. The results of such an experiment are included in Table I. It is seen here that passive immunization 3 days after first giving antigen prevents acquisition of the capacity for a subsequent response in the presence of antibody despite the fact that a peak primary direct PFC response has occurred.

The effect of passive immunization 3 days after primary immunization was further characterized by comparing the later phases of the primary response of
TABLE I

Early and Late Passive Immunization. Effects on Primary and Secondary Responses

| Day | PFC/spleen* | PFC/spleen* |
|-----|-------------|-------------|
|     | -- SRBC†   | 122,650 (60,200) |
|     | -- SRBC‡   | 550 (0) |
|     | -- SRBC§   | 109,300 (0) |
|     | -- SRBC‡   | PI§ SRBC§ 84,400 (131,950) |
|     | -- SRBC‡   | PI§ SRBC‡ 2,300 (0) |
|     | -- SRBC‡   | PI§ SRBC‡ 4,150 (0) |

* Mean group response of six to eight mice, rounded to nearest 50; direct PFC with indirect PFC in parentheses.
† 1.5 × 10⁶ sheep erythrocytes injected intravenously.
‡ 0.4 ml of late-immune mouse anti-SRBC serum administered intraperitoneally.

Fig. 1. The effect of passive immunization 3 days after primary active immunization on the primary response to 1.5 × 10⁶ intravenous SRBC and the response to a second similar SRBC injection which is immediately preceded by passive immunization. Each point is the mean response of six to eight mice. The group range is shown by dotted vertical lines for direct, and horizontal bars for indirect PFC. The lower graph is derived from a comparison of group means. Passive immunization (PI) represents 0.4 ml of intraperitoneal late-immune mouse anti-SRBC serum.
mice so treated with that of matched groups receiving active immunization alone. Levels of both direct and indirect PFC in the spleen were measured at various times up to 21 days after primary immunization. Two additional effects of passive immunization 3 days after primary immunization were seen: (a) the

### TABLE II

*Effect on Secondary Response of Passive Immunization at Varying Times after Primary Active Immunization*

| SRBC* | PI‡ | PFC/spleen§ day 25 |
|-------|-----|--------------------|
|       |     | Direct | Indirect          |
|       |     | (day)  | (day)             |
| 0 and 21 | 20  | 127,350 (83,000–152,500) | 163,400 (121,000–247,500) |
| 0 and 21 | 3 and 20 | 9,150 (2,350–15,750) | 0 |
| 0 and 21 | 5 and 20 | 16,400 (11,300–24,100) | 10,450 (5,600–14,700) |
| 0 and 21 | 7 and 20 | 49,300 (28,500–70,000) | 61,200 (41,500–94,000) |
| 0 and 21 | 10 and 20 | 133,500 (78,000–164,000) | 171,350 (105,000–281,000) |

* 1.5 X 10^8 sheep erythrocytes injected intravenously.
† 0.4 ml of late-immune mouse anti-SRBC serum administered intraperitoneally.
§ Mean group response of six mice, rounded to nearest 50, with range in parentheses.

### TABLE III

*Effect of Passive Immunization on Response to Multiple SRBC Injections*

| Day 0 | Day 3–21 | Day 25 |
|-------|----------|-------|
|       |          | PFC/spleen* |
|       |          | Direct | Indirect |
| SRBC‡ | PI§ + SRBC‡ twice weekly | 38,100 (24,000–61,400) | 0 |
| SRBC‡ | SRBC‡ twice weekly | 40,800 (30,800–48,400) | 140,200 (125,800–161,400) |

* Mean response of group of eight mice; range in parentheses; assay 4 days after last SRBC injection.
† 1.5 X 10^8 sheep erythrocytes injected intravenously.
§ 0.4 ml of late-immune mouse anti-SRBC serum administered intraperitoneally starting on day 3, each followed in 24 hr by SRBC.

procedure prevented the appearance of the indirect PFC component of the primary response, (b) it resulted in a more rapid decline of the peak direct PFC response (Fig. 1).

Passive immunization 3 days after primary immunization prevented acquisition of the capacity for a subsequent response in the presence of antibody. It was tested to what extent such an effect was demonstrable when passive immunization was further delayed. The results shown in Table II indicate a decreasing
effect which is still demonstrable after a delay of as much as 7 days. Thus passive immunization given after the appearance and even after the peak of the primary indirect PFC response (Fig. 1) can still affect the development of the capacity for a subsequent response in the presence of antibody.

Finally, the response to a course of twice weekly injections of SRBC, each given 24 hr after anti-SRBC serum, and begun 4 days after primary immunization, was measured.

In these circumstances a level of direct PFC response similar to that of mice receiving equivalent active immunization only was present after 25 days (Table III). The mice receiving passive immunization had not however generated any indirect PFC by this time, indicating that escape from antibody inhibition was possible in the absence of an indirect PFC response.

DISCUSSION

It is recognized that primary and secondary responses may have apparent distinguishing features contributed to by differences in the composition of the cellular populations available for the response to antigen (6). Distinctions between primary and secondary responses are sometimes muddied but can often be drawn with respect to parameters such as rapidity, magnitude and antigen sensitivity, class and combining affinity of immunoglobulin produced, sequence of immunoglobulin production, effect of adjuvants, and susceptibility to a variety of suppressants including antibody to the antigen, anti-lymphocyte antibody, and X-irradiation (7-18). The ability to mount a secondary response may be ascribed to immunological memory. Data previously presented concerning the response to SRBC is in keeping with the view that immunization results in two types of memory. The first is based on augmentation of the original system for response, is present from a very early time after immunization, and is well demonstrated when the response of immune cells is looked for after transfer into a milieu free of antibody to SRBC. The second appears later and is necessary for the normal secondary response in vivo. It is presumably dependent on a change occurring in the composition of the cell populations available for response to antigen, one effect of which is a different type of in vitro response becoming apparent about 10 days after in vivo immunization (5). The experiments presently reported further examine this second form of immunological memory. In the system used a single, easily ascertainable feature, the appearance of a response which is not dramatically suppressed by the presence of antibody to the antigen, is chosen to indicate the presence of such memory.

It was found that passive immunization, with late-immune anti-SRBC serum on the 3rd day of the primary response to $1.5 \times 10^6$ SRBC, did not depress the subsequent peak direct PFC response but prevented establishment of memory. A concomitant effect was the abolition of the primary indirect PFC response. These results suggested two questions: (a) was the abolition of the indirect PFC
response effected by preventing transformation of direct PFC to indirect PFC or by suppression of a separate group of responding cells and \( b \) is the appearance of indirect PFC synonymous with the establishment of immunological memory.

With respect to the first question, such data as we presently have suggests that the normal decline of direct PFC is not due to their transformation into indirect PFC. Thus, \( a \) the decline in splenic direct PFC is accelerated when appearance of indirect PFC is prevented by late passive immunization; \( b \) spleen cell cultures set up at the time transformation should be occurring failed to yield detectable numbers of indirect PFC \( (5) \); \( c \) the in vitro response of cells from passively immunized animals suggests that antibody does not suppress the direct PFC response by incorporation into or on cells \( (5) \). If so, at the time of delayed passive immunization the affected process is probably still dependent on stimulation by extracellular antigen rather than one triggered in an earlier phase of the primary response.

With respect to the second question, the appearance of indirect PFC was not synonymous with the establishment of memory. The capacity for a subsequent response in the presence of anti-SRBC antibody was still significantly crippled when passive immunization was delayed until the time of the peak primary indirect PFC response. Again, mice given passive immunization on day 3 of the primary response and further combined active and passive immunization twice weekly starting on day 4, generated by day 25 similar numbers of splenic direct PFC as mice receiving identical active immunization but no antibody. The passively immunized mice had not, however, produced detectable indirect PFC. Under the conditions of this experiment, mice which had not had indirect PFC responses evidenced postprimary direct PFC responses of the same magnitude as those shown by mice which had produced indirect PFC responses.

**SUMMARY**

The acquisition of a capacity to respond well to sheep erythrocytes in the presence of anti-SRBC antibody was taken as an indication of the presence of immunological memory. By the use of passive immunization, both the primary IgG plaque-forming cell response and the establishment of memory were abolished, despite occurrence of a full peak IgM PFC response. Evidence for regarding the acquisition of memory and the IgM PFC and IgG PFC responses as three separate processes was presented. Antibody on day 3 of the response to \( 1.5 \times 10^9 \) SRBC abolished formation of memory; this effect was less if passive immunization was further delayed and absent by day 10.

The technical assistance of Miss Sandra Struthers is gratefully acknowledged.

**BIBLIOGRAPHY**

1. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Washington)*. 140:405.
2. Jerne, N. K., A. A. Nordia, and C. Henry. 1963. The agar plaque technique for recognizing antibody-producing cells. *In Cell Bound Antibodies*. B. Amos and H. Koprowski, editors. Wistar Institute Press, Philadelphia, Pa. 109.

3. Dresser, D. W., and H. H. Wortis. 1965. Use of an antiliglobulin serum to detect cells producing antibody with low haemolytic efficiency. *Nature (London).* 208:859.

4. Wortis, H. H., R. B. Taylor, and D. W. Dresser. 1966. Antibody production studied by means of the LHG assay. I. The splenic response of CBA mice to sheep erythrocytes. *Immunology.* 11:503.

5. Radcliffe, G. N., and M. A. Axelrad. 1971. Immunological memory in vitro. *J. Exp. Med.* 133:846.

6. Sterzl, J., and A. M. Silverstein. 1967. Developmental aspects of immunity. *Adv. Immunol.* 6:337.

7. Glenny, A. T., and H. J. Sfidmersen. 1921. Notes on the production of immunity to diphtheria toxin. *J. Hyg.* 20:176.

8. Uhr, J. W., M. S. Finkelstein, and J. B. Baumann. 1962. Antibody formation. III. The primary and secondary antibody response to bacteriophage φX 174 in guinea pigs. *J. Exp. Med.* 115:655.

9. Uhr, J. W., and M. S. Finkelstein. 1963. Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage φX 174. *J. Exp. Med.* 117:457.

10. Svehag, S. E., and B. J. Mandel. 1964. The formation and properties of poliovirus-neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, amnesia, and sensitivity to X-irradiation. *J. Exp. Med.* 119:21.

11. Plotz, P. H., N. Talal, and R. Asofsky. 1968. Assignment of direct and facilitated haemoletic plaques in mice to specific immunoglobulin classes. *J. Immunol.* 100:744.

12. Fecsik, A. I., W. T. Butler, and A. H. Coons. 1964. Studies on antibody production. XI. Variation in the secondary response as a function of the length of the interval between two antigenic stimuli. *J. Exp. Med.* 120:1041.

13. Steiner, L. A., and H. N. Eisen. 1967. Sequential changes in the relative affinity of antibodies synthesized during the immune response. *J. Exp. Med.* 126:1161.

14. Gabrielson, A. E., and R. A. Good. 1967. Chemical suppression of adaptive immunity. *Adv. Immunol.* 6:91.

15. Rowley, D. A., F. W. Fitch, M. A. Axelrad, and C. W. Pierce. 1969. The immune response suppressed by specific antibody. *Immunology.* 16:549.

16. Rowley, D. A., and F. W. Fitch. 1968. Clonal selection and inhibition of the primary antibody response by antibody. *In Regulation of the Antibody Response*. B. Cinader, editor. Charles C Thomas, publisher, Springfield, Ill. 127.

17. Denman, A. M. 1969. Antilymphocytic antibody and auto-immune disease: a review. *Clin. Exp. Immunol.* 5:217.

18. Humphrey, J. H. 1965. The suppression of immune responses by non-specific agents. *In Immunological Diseases*. M. Samter, editor. Little, Brown and Co., Boston, Mass. 100.