THE X-Y-Z SCHEME OF IMMUNOCYTE MATURATION

VII. Cell Division and the Establishment of Short-Term IgM Memory

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Immune memory has been explained according to three basic schemes: (*a*) altered handling of antigen due to combination with antibody or due to the ability of macrophages to degrade antigen more effectively; (*b*) expansion of a clone of reactive cells, the daughter cells being identical to the original antigen-sensitive cells (1–3); and (*c*) differentiation of a unique class of "memory cells" which react more rapidly and effectively on antigen contact than the original antigen-sensitive cells (4–7). In this paper we examine the establishment of short-term memory to distinguish between these hypotheses. We define memory as the capacity to give a greater and more rapid response to a second injection of antigen than to a first injection. In the response of mice to sheep erythrocytes (SRBC), measured by the hemolytic plaque assay (8), early IgM memory arises within the 1st day, approximately 2 days before any substantial increase in direct plaques (9). IgM memory continues to increase during the 1st wk after the primary injection (9, 10).

We sought to determine whether this early establishment of memory could occur in the presence of an antimetabolite which prevents cell division by inhibiting thymidine synthesis, methotrexate (11). The results indicate that antigen-sensitive cells undergo a qualitative change during the 1st day after primary stimulation and, on further antigen contact, begin to divide. There is no such nonproliferative lag phase after a secondary injection.

Materials and Methods

Mice.—A/Jax mice were bred in our laboratory from Jackson Laboratories stock (Bar Harbor, Me.). Animals of both sexes, 6–10 wk old, were used in each group.

Antigen.—Sheep blood in Alsever's solution was purchased from Mission Laboratory Supply, Inc., Rosemead, Calif., and used within 3 wk. Erythrocytes were washed and resuspended in saline, then injected into the retroorbital plexus (12).

Drugs.—Methotrexate, sodium (American Cyanamid Co., Lederle Laboratories Division, Pearl River, N.Y.) 25 mg/kg body weight and folinic acid (leucovorin, Lederle) 25–35 mg/kg were injected intraperitoneally. Hydroxyurea (Nutritional Biochemicals Co., Cleveland, Ohio) was used in doses of 0.05–2.5 mg/kg, injected intraperitoneally.

Preparation of Cell Suspensions.—Mice were killed, their spleens were removed and minced

with scalpels in Eagle's minimal essential medium without bicarbonate (*p*H adjusted to 7.4 with NaOH). The suspension was passed through two stainless steel screens, 80 and 250 mesh, respectively. Nucleated cells were counted in a hemacytometer after dilution into a hemolytic agent, 0.01% gentian violet, 2% acetic acid. In most experiments, the total volume of the cell suspension was recorded in order to calculate the plaques per spleen.

Hemolytic Assays.—Hemolytic plaque assays were performed by a modification of the technique of Jerne and Nordin (8). To 0.75 ml 0.7% agarose, dissolved in Eagle's minimal essential medium, were added 0.04 ml 20% washed sheep erythrocytes, 0.04 ml guinea pig serum (complement), and a variable amount of spleen cell suspension. This mixture was poured onto a 1.4% agarose-Eagle's minimal essential medium underlayer in a 60 mm Petri dish and incubated 2–3 hr at 37°C. Plaques were counted by indirect light.

RESULTS

Early IgM Memory.—Memory is defined as the number of plaques resulting from two injections of antigen minus the sum of the plaques expected from either injection alone. Our standard dose regimen was an injection of 2×10^6 SRBC ("low" dose), followed at varying intervals by an injection of 4×10^8 SRBC ("high" dose). The total response and the calculated memory for several dose regimens are shown in Table I. The primary response to either the high or the low dose alone is included for reference. Also included are our code designations for each group.

During the first 2 days of the response to either dose alone, there is little increase in the number of direct plaques (see groups 2L and 2H in Table I). During this period, however, the mice have become able to respond much more rapidly and vigorously to a second dose of antigen; that is, they have been primed. Comparing group 1,2 with groups receiving only the primary injection (3L) or only the secondary injection (2H), it is apparent that the injections elicit a far greater response in combination than alone. The memory increases further during the 2nd day after primary injection: the memory value is larger for group 2,2 than for group 1,2 (see reference 9).

Cell Division and the Immune Response.—We employed methotrexate (MTX), a folic acid antagonist, to prevent cell division. Folinic acid (leucovorin = LV) was injected 24 hr after MTX in an attempt to prevent further inhibition and increase the general health of the animals, although most of the MTX should have been excreted by this time (13). LV is an effective antidote when administered simultaneously with MTX but has little effect when administered 1 day later (Table II, and reference 14).

There is a striking contrast between the first and subsequent days of the response of mice to SRBC. MTX administered simultaneously with antigen (or before antigen) does not inhibit the response to a high dose of SRBC (Table II). However, MTX administered after 1 or 2 days have elapsed greatly diminishes the plaque number. The same disparity between the first and subsequent days is apparent in the response to a low dose of SRBC; MTX administered

TABLE I

The Plaque Response to a Low Dose and a High Dose of SRBC, and Combinations Thereof, with Calculations of Immune Memory*

Code‡	Primary dose	Time between injections	Secondary dose	Time between last injection and assay	No. of mice	Plaques/10^6 spleen cells		Memory§
						Mean logs	Antilog	Plaques/10^6 spleen cells
		days		*days*				
Background								
—, —	—	—	—	—	5	0	1	—
Low primary								
2L	Low	—	—	2	5	1.0	2	—
3L	Low	—	—	3	10	1.0	2	—
4L	Low	—	—	4	14	4.4	21	—
5L	Low	—	—	5	10	5.7	51	—
High primary								
2H	High	—	—	2	10	1.5	2.8	—
3H	High	—	—	3	34	5.3	39	—
4H	High	—	—	4	13	8.0	256	—
Secondary								
1,1	Low	1	High	1	6	2.1	4.3	2
1,2	Low	1	High	2	19	5.3	39	35
1H,2	High	1	High	2	8	6.5	90	49
1,3	Low	1	High	3	5	7.6	194	134
2,1	Low	2	High	1	8	2.8	7	5
2,2	Low	2	High	2	35	6.8	111	88
Multiple								
3 Daily low doses					7	4.4	21	17
4 Daily low doses					9	6.9	119	94

* A low dose is 2×10^6 SRBC. A high dose is 4×10^8 SRBC.

‡ In a series, the primary injection is a low dose and the secondary, high, unless otherwise indicated. For example, mice in group 2,2 received 2×10^6 SRBC on day 0, 4×10^8 SRBC on day 2, and were killed day 4.

§ "Memory" was calculated by subtracting from the plaques obtained the sum of all plaques expected as a result of either injection alone. This is not applicable in groups receiving only one injection.

simultaneously with antigen (on day 0) has no effect, but MTX administered on day 1, 2, or 3 decreases the 4-day plaque response 8- to 15-fold (Fig. 1, A).

Cell Division and the Establishment of Memory.—The effect of MTX on the establishment of short-term memory was examined in mice which received a second antigenic challenge 2 days after the primary injection. MTX was administered on day 0, 1, 2, or 3 relative to the primary injection, and the response was measured on day 4 (Fig. 1, B). Two conclusions can be drawn from this experiment. (*a*) MTX does inhibit the events occurring within 24 hr after a secondary injection of antigen. This is in contrast to its lack of effect during the 1st day of a primary response. (*b*) Priming, like the primary response itself, be-

TABLE II

Effect of Methotrexate on the 3-Day Response of Mice to a High Dose of Sheep Erythrocytes*

Drug administration‡	Plaques/10^6 spleen cells		No. of mice
	Mean $\log_2$	Antilog	
No drug	5.4	42	19
Experimental groups			
MTX day 0, LV day 1	5.1	34	10
MTX day 1, LV day 2	0.7	1.6	14
MTX day 2, LV 4 hr before death	3.9	15	7
Control groups			
MTX + LV, day 2	4.8	28	13
LV day 0	4.8	28	5
MTX 1 day before SRBC, LV day 0	6.8	111	10
MTX day 1, no LV	1.2	2.3	5

* All mice received 4×10^8 SRBC on day 0.

‡ Methotrexate, 25 mg/kg body weight; and leucovorin, 25–35 mg/kg.

comes susceptible to MTX 1 day after the initial antigen injection. MTX administered at the time of the initial injection had no effect on the ability of mice to respond to a second dose of antigen, but MTX injected 1 day after antigen diminished the response to a second injection given on day 2.

The experiment shown in Fig. 1, B suggested that priming has two phases: a nonproliferative phase, approximately 1 day long, followed by a proliferative phase. To confirm the nonproliferative nature of the first phase, we altered the regimen, administering the second injection of SRBC just 1 day after the primary injection. When MTX was administered along with the primary injection, the 1 day priming was not inhibited (Table III).

Although there is no doubt that MTX inhibits proliferation, its metabolic effects are not restricted to inhibition of thymidine synthesis (11). To support our contention that the inhibitory effect of MTX is due to prevention of cell

division, we did some preliminary experiments employing hydroxyurea, a drug which inhibits DNA synthesis quite specifically (15). Hydroxyurea mimics the

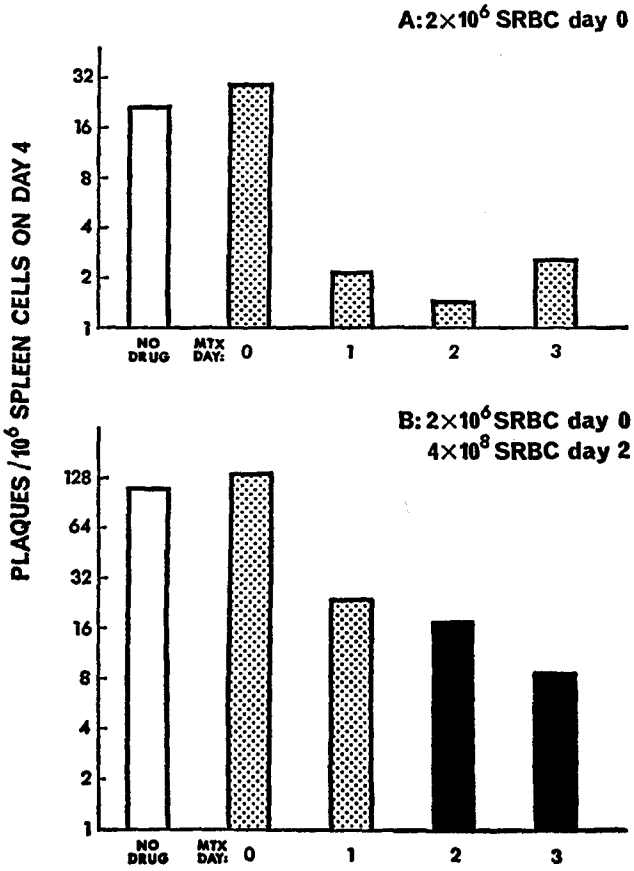

FIG. 1, A and B. Effect of methotrexate (MTX) on the primary and secondary responses, plaques per million nucleated spleen cells on day 4. Mice received MTX (25 mg/kg) on the day indicated on the abscissa, and leucovorin (35 mg/kg) 24 hr later.

Fig. 1, A. *Primary response* to 2×10^6 sheep erythrocytes.

FIG. 1, B. *Secondary response.* Mice received 2×10^6 SRBC on day 0 and 4×10^8 SRBC on day 2. The clear bars indicate controls not injected with drugs; hatched bars indicate animals treated with MTX after a primary antigen injection; and, black bars indicate animals treated with MTX after a secondary antigen injection.

effects of MTX: it inhibits the secondary response but is without effect on the 1st day of priming.

Specificity of Memory.—To show that memory was specific for the priming antigen we performed the experiments presented in Table IV. Preinjection with

burro erythrocytes, which cross-react only slightly with sheep or goat erythrocytes, has no effect on the subsequent 2-day response to sheep RBC (Table IV,

TABLE III

*Lack of Effect of Methotrexate on 1-Day Priming**

Drug administration	Plaques/10^6 spleen cells		No. of mice
	Mean log$_2$	Antilog	
None	5.3	39	19
MTX day 0‡	5.0	32	17

* Both groups of mice received 2×10^6 SRBC on day 0 and 4×10^8 SRBC on day 1. The plaque assay was performed on day 3.

‡ Mice received 25 mg/kg body weight methotrexate, intraperitoneally, at the same time as the first antigen injection, and 35 mg/kg leucovorin a few hours before the second injection of SRBC.

TABLE IV

Antigen Specificity of Short-Term Memory

Antigen injections		Type of RBC used in assay	Plaques/10^6 spleen cells on day 4		Memory
Day 0: 2×10^8 RBC	Day 2: 4×10^8 RBC		Mean log$_2$	Antilog	Plaques/10^6 spleen cells
*Experiment 1**					
(*a*) —	Sheep	Sheep	2.0	4.0	—
(*b*) Burro	Sheep	Sheep	2.7	6.5	2.5
(*c*) Burro	Burro	Sheep	0.8	1.7	0
(*d*) Burro	Burro + sheep	Sheep	4.3	20.0	14.0
*Experiment 2**					
(*e*) —	Goat	Goat	4.0	16.0	—
(*f*) Burro	Burro + goat	Goat	4.6	24.0	6.0
For reference					
(*g*) Sheep	Sheep	Sheep	6.8	110.0	95.0

* There were five to seven mice in each group.

a and *b*). During a specific secondary response to burro erythrocytes, there is a slight stimulation of the response to sheep or goat erythrocytes (Table IV, *d* and *f*). The increment is small, however, compared with specific memory (Table IV, *g*).

Time of Assay.—After a short priming interval, the secondary IgM response

reaches a very sharp peak between the 2nd and 3rd days (9), a fact which some have overlooked. To avoid inaccuracy caused by missing this sharp peak, we measured the response before the peak in all cases. Thus we may be measuring the delay or diminution of the peak response, or some combination of the two. Both effects have been noted by Rivarola, et al. (16) depending upon the dose regimen of MTX. In limited experiments, we found no evidence of delay. Either delay or diminution, however, would be a valid measure of inhibition of the response.

DISCUSSION

During the 1st day after primary antigen injection, a mouse does not produce detectable plaque-forming cells, but it does develop the ability to respond with rapid proliferation and antibody production to a second injection of antigen. Methotrexate has no effect on the priming which occurs during the 1st day. During the 2nd day after injection of SRBC, the short-term memory continues to increase; this increase can be inhibited by MTX. Priming thus has two phases: the first, comprising approximately 1 day, does not require cell division, but rather some qualitative change in cells; the second consists of proliferation of some or all of these altered cells.

The phases which we have measured separately as the responses to two injections of antigen may be identical to those normally occurring in the primary response. The greater rapidity and magnitude of the primary response to a large dose of antigen (Table I, and references 9 and 17) can be explained as follows. The first contact of antigen-sensitive cells (X cells) with antigen leads to development of cells (Y cells) which are capable of reacting to further antigen contact by very rapid proliferation and production of antibody (9). In our system, the opportunity for further antigen contact is minimal after the *low* primary dose, unless a second injection is given. After the *high* primary dose, enough antigen will remain to induce nearly maximal division and antibody production. This view is supported by our findings, shown in Table I, that (*a*) mice receiving a second dose 1 day after the low primary dose respond as well as animals receiving the high primary dose; (*b*) a second injection 1 day after the *high* primary injection causes only a small enhancement of the response; and (*c*) even the low doses, if given daily, can induce a vigorous response. None of these findings is predicted by the hypothesis that cells, once triggered, respond in a predetermined manner for a predetermined time and can no longer be affected by antigen contact. Clearly, the IgM-producing cell is still responsive to further antigenic contact (19, 20, 10). An injection just before the peak of a secondary IgM response can prolong the increase of IgM plaques (21).

Radioautographic examination of plaque-forming cells which have been exposed to isotopically labeled thymidine in vitro or in vivo have led to various conclusions. Some authors have concluded that a large proportion of PFC pres-

ent 3 or 4 days after antigenic stimulation have not synthesized a complement of DNA at any time since the antigen administration (22, 23). Other studies have indicated that all plaque-forming cells arise from dividing precursors after the 2nd day (24, 25). Most of these studies have not been designed to measure the rate of division. Those which have attempted to measure the cell cycle time have not agreed: Koros et al. (18) found a generation time of approximately 7 hr, while Tannenberg calculated 13 hr (26).

Studies utilizing inhibitors of replication are difficult to interpret, owing to the problems of dosage, morbidity, and indirect effects. We have tried to avoid this problem insofar as possible by limiting the duration of the drug effect. Most inhibition studies indicate that cell division is necessary for the increase in plaque-forming cells (27–30). Rowley et al. measured the disappearance of plaques after administration of vinblastine or colchicine, and found a cell cycle time, approximately equal to the plaque number doubling time, of 5–10 hr after the 2nd day of the response (31). Perhaps the clearest demonstration that cell division is required for the immune response is that of Dutton and Mishell (32) who caused dividing cells to incorporate a lethal dose of tritiated thymidine, thereby preventing a response by cultured mouse spleen cells, unless the "hot pulse" was restricted to the 1st day after antigen administration. Uyeki (33) showed that MTX inhibits the response in vivo if given on the 2nd day after antigen administration, but not if given along with antigen, and we have found the same.

Our evidence indicates a qualitative difference between the cells which respond to primary and secondary injections; i.e., X and Y cells. On the basis of entirely different evidence, others have come to the same conclusion (34, 35). We have found that a second administration of antigen within a few days after the priming dose causes plaque-forming cells to increase rapidly. The considerable increase which occurs within 1 day is due to proliferation, as it is inhibited by methotrexate. On the contrary, events of the 1st day after the *primary* injection of SRBC are not inhibited by methotrexate nor by hydroxyurea.

Apparently, the preparatory events of the 1st day postprimary need not be repeated after the second injection. These events undoubtedly include macrophage processing and distribution of antigen, although these processes probably require only a short time, on the order of 1 hr (36–38). Other events may include preparation for division, interaction of thymus-derived and bone marrow-derived cells (39), and morphological changes (40, 41). We have suggested that (additional) receptor molecules synthesized during the 1st day might mediate an interaction of cells and antigen leading to immediate proliferation (9).

We emphasize that the immediate proliferative response to reinjection of antigen applies to short-term IgM memory only. Short-term memory wanes approximately 1 month after priming (9, 10). Recently described phenomena in other systems (42, 43) parallel our finding, but are not exactly comparable.

However, the results of O'Brien and Coons (44) and our own preliminary results indicate that the secondary response after long priming intervals includes a nonproliferative lag phase. We are currently engaged in experiments designed to confirm or deny the existence of two varieties of IgM memory, and to compare IgM and IgG memory.

SUMMARY

Short-term IgM memory is established within 1 day after primary injection. This 1 day priming is independent of cell division as it can take place in the presence of methotrexate or hydroxyurea.

The primary response involves a similar 1 day nonproliferative phase. On the contrary, cells responding to a second injection of antigen after short priming intervals begin to proliferate within hours. This implies a qualitative difference between the antigen-sensitive cells in primed and normal animals.

After 1 day, further proliferative expansion of memory can occur, which is probably antigen dependent. The response to several dose regimens indicates that repeated antigenic contact is needed to maintain the IgM response.

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BIBLIOGRAPHY

1. Burnet, F. M. 1959. The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville.
2. Lederberg, J. 1959. Genes and antibodies. *Science* **129**:1649.
3. Gowans, J. L., and J. W. Uhr. 1966. The carriage of immunological memory by small lymphocytes in the rat. *J. Exp. Med.* **124**:1017.
4. Leduc, E. H., A. H. Coons, and J. M. Connolly. 1955. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exp. Med.* **102**:61.
5. Sercarz, E., and A. H. Coons. 1962. The exhaustion of specific antibody producing capacity during 2° response. *In* Mechanisms of Immunological Tolerance. M. Hašek, A. Lengerova, and M. Vojtiškova, editors. Czechoslovak Academy of Sciences, Prague. 73.
6. Nossal, G. J. V. 1965. The mechanism of action of antigen. *Australas. Ann. Med.* **14**:321.
7. Jílek, M., and J. Šterzl. 1966. Influence of the amount of antigen and the interval on the secondary reaction. *Folia Microbiol.* **12**:6.
8. Jerne, N. K., and A. A. Nordin. 1963. Plaque-formation by single antibody-producing cells in agarose gel. *Science.* **140**:405.
9. Sercarz, E., and V. S. Byers. 1967. The X-Y-Z scheme of immunocyte maturation. III. Early IgM memory and the nature of the memory cell. *J. Immunol.* **98**:836.

10. Wigzell, H. 1966. The rise and fall of 19S immunological memory against sheep red cells in the mouse. *Ann. Med. Exp. Biol. Fenn.* **44**:209.
11. Bertino, J. R. 1963. The mechanism of action of the folate antagonists in man. *Cancer Res.* **23**:1286.
12. Pinkerton, W., and M. Webber. 1964. A method of injection of small laboratory animals by the ophthalmic plexus route. *Proc. Soc. Exp. Biol. Med.* **116**:959.
13. Henderson, E. S., R. H. Adamson, C. Denham, and V. T. Oliverio. 1965. The metabolism of tritiated methotrexate. I. Absorption, excretion, and distribution in mice, rats, dogs and monkeys. *Cancer Res.* **25**:1008.
14. Berenbaum, M. C., and I. N. Brown. 1965. The effect of delayed administration of folinic acid on immunological inhibition by methotrexate. *Immunology.* **8**:251.
15. Schwartz, H. S., M. Garofalo, S. S. Sternberg, and F. S. Phillips. 1965. Hydroxyurea: Inhibition of deoxyribonucleic acid synthesis in regenerating liver of rats. *Cancer Res.* **25**:1867.
16. Rivarola, A., M. Friedman, and W. Lawrence, Jr. 1967. Methotrexate and the immune response. *Transplantation.* **5**:1223.
17. Wigzell, H., G. Möller, and B. Andersson. 1966. Studies at the cellular level of the 19S immune response. *Acta Pathol. Microbiol. Scand.* **66**:530.
18. Koros, A. M. C., H. Fuji, and N. K. Jerne. 1966. Kinetics of proliferation of clones of antibody-producing cells. *Fed. Proc.* **25**:305.
19. 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.
20. Svehag, S. E., and B. Mandel. 1964. The formation and properties of poliovirus-neutralizing antibody. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction. *J. Exp. Med.* **119**:1.
21. Sercarz, E. 1968. Regulatory role of antigen in determining the quality of an antibody response. *Fed. Proc.* **27**:493.
22. Šterzl, J., J. Vešely, M. Jílek, and M. Mandel. 1965. Differentiation of immunologically competent cells. *In* Genetic Variations of Somatic Cells. J. Klein, M. Vojtiškova, and V. Zeleny, editors. Czechoslovak Academy of Sciences, Prague. 233.
23. Tannenberg, W. J. K. 1967. Induction of 19S antibody synthesis without stimulation of cellular proliferation. *Nature.* **214**:293.
24. Ellis, S. T., J. L. Gowans, and J. C. Howard. 1967. Cellular events during the formation of antibody. *Cold Spring Harbor Symp. Quant. Biol.* **32**:395.
25. Szenberg, A., and A. J. Cunningham. 1968. DNA synthesis in the development of antibody-forming cells during the early stages of the immune response. *Nature.* **217**:747.
26. Tannenberg, W. J. K., and A. N. Malaviya. 1968. The life cycle of antibody-forming cells. I. The generation time of 19S hemolytic plaque-forming cells during the primary and secondary responses. *J. Exp. Med.* **128**:895.
27. Syeklocha, D., L. Siminovitch, J. E. Till, and E. A. McCulloch. 1966. The proliferation state of antigen-sensitive precursors of hemolysin-producing cells determined by the use of the inhibitor, vinblastine. *J. Immunol.* **96**:472.

28. Frisch, A. W., G. W. Davies, and V. Milstein. 1963. The inhibition of hemagglutinin formation in mice by thioguanine. *J. Immunol.* **89**:300.
29. Berenbaum, M. C. 1966. Role of mitosis and mitotic inhibition in the immunosuppressive action of thioguanine. *Nature.* **210**:41.
30. Schwartz, R. S. 1965. Immunosuppressive drugs. *Progr. Allergy.* **9**:246.
31. Rowley, D. A., F. W. Fitch, D. E. Mosier, S. Solliday, L. W. Coppleson, and B. W. Brown. 1968. The rate of division of antibody-forming cells during the early primary immune response. *J. Exp. Med.* **127**:983.
32. Dutton, R. W., and R. I. Mishell. 1967. Cell populations and cell proliferation in the *in vitro* response of normal mouse spleen to heterologous erythrocytes. *J. Exp. Med.* **126**:443.
33. Uyeki, E. M. 1967. Effects of several antitumor agents on spleen hemolysin plaque-forming cells. *Biochem. Pharmacol.* **16**:53.
34. Rowley, D. A., and F. W. Fitch. Clonal selection and inhibition of the primary antibody response by antibody. *In* Regulation of the Antibody Response. B. Cinader, editor. Charles C. Thomas, Springfield, Ill. 127.
35. Modabber, F. 1968. An antigen-binding technique at the molecular level for the direct study of the immune memory cell. Ph.D. Thesis. University of California, Los Angeles.
36. Fishman, M., and F. L. Adler. 1963. Antibody formation initiated *in vitro*. II. Antibody synthesis in x-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen. *J. Exp. Med.* **117**:595.
37. Askonas, B. A., and F. L. Rhodes. 1965. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. *Nature.* **205**:470.
38. Wust, C. J. 1967. Clearance of sheep and rat erythrocytes in the mouse: selenium-75 as a marker. *RES—J. Reticuloendothel. Soc.* **4**:43.
39. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. *Proc. Nat. Acad. Sci. U. S. A.* **59**:296.
40. Haskill, J. S. 1967. Density distribution analysis of antigen sensitive cells in the rat. *Nature.* **216**:1229.
41. Raidt, D. J., R. I. Mishell, and R. W. Dutton. 1968. Cellular events in the immune response. Analysis and *in vitro* response of mouse spleen cell populations separated by differential flotation in albumin gradients. *J. Exp. Med.* **128**:681.
42. Vischer, T. L., and P. Stastny. 1967. Time of appearance and distribution of cells capable of secondary immune response following primary immunization. *Immunology.* **12**:675.
43. Jacobsen, E. B., and G. J. Thorbecke. 1968. Relationship of germinal centers in lymphoid tissue to immunologic memory. III. Proliferative response of primed cells from splenic white and red pulp following reexposure to antigen *in vitro*. *J. Immunol.* **101**:515.
44. O'Brien, T. F., and A. H. Coons. 1963. Studies on antibody production. VII. The effect of 5-bromodeoxyuridine on the *in vitro* anamnestic antibody response. *J. Exp. Med.* **117**:1063.