THE X-Y-Z SCHEME OF IMMUNOCYTE MATURATION

IV. The Exhaustion of Memory Cells

By VERA S. BYERS and ELI E. SERCARZ, Ph.D.

(*From the Department of Bacteriology, University of California, Los Angeles 90024*)

(Received for publication 25 September 1967)

The response of an animal to antigen is altered after its first exposure. The intensified secondary response has been attributed by some to the presence of qualitatively different "memory" cells. The concept of two types of antigen-sensitive cells, occurring as successive stages in immunocyte maturation, was elaborated by Burnet (1) and Leduc *et al.* (2), and later into an X-Y-Z scheme by Sercarz and Coons (3). In the X-Y-Z formulation, the X cell is the first antigen-sensitive cell in the lymphocytic series, which upon stimulation is converted into a Y or memory cell. Triggering of Y cells by antigen results in division and irreversible maturation to a plasmacytic series of Z cells whose terminal member is the mature, antibody-producing plasma cell.[1] Šterzl also uses this terminology (4).

Two major states of antigen-induced unresponsiveness have been described; immune paralysis or tolerance (5, 6) and immune exhaustion (4). Although unresponsiveness in both immunologically naive and experienced animals has been documented and discussed, the two categories have never been clearly distinguished. Paralysis may be defined as complete and specific inhibition of immune responsiveness by a particular antigen; exhaustion, as the productive expenditure of a particular compartment of immunocytes. Empirically, paralysis should ensue with no detectable antibody formation, while exhaustion should be characterized by a period of significant antibody production followed by a transient state of unresponsiveness, until new antigen-sensitive cells are recruited.

According to this hypothesis, four possible cellular types of unresponsiveness may be induced by antigen: paralysis of X cells, exhaustion of X cells, paralysis of Y cells, and exhaustion of Y cells. In some cases, two of these could coexist. We have undertaken (*a*) to investigate exhaustion at both the X cell and the Y cell level, and (*b*) to determine whether exhaustion is an indissociable component of paralysis induction.

(*a*) Early experiments, which showed that continued administration of antigen did not lead to hyperimmunization but rather to a diminished state of reactivity, (7–10) seemed to favor the possibility of concurrent X and Y exhaustion. These papers suggested the cell-transfer experiment of Sercarz and Coons, which showed that for a short time following a secondary antibody response, cells transferred to a normal recipient were unable to produce a further response upon challenge. It was concluded that triggering the Y→Z conversion in the donor animals resulted in a temporary

[1] Sercarz, E. The X-Y-Z scheme of immunocyte maturation. I. Immune paralysis, exhaustion, and memory. Submitted for publication.

paucity of memory (Y) cells, rendering the transferred population incapable of further response (11). However recent experiments based on the exhaustion concept have not attempted to differentiate X cell from Y cell exhaustion.

(*b*) Several reports have indicated a finite time for induction of paralysis (12, 13). These results clearly imply that under certain experimental conditions antibody formation occurs in some cells (X or Y) concomitant with paralysis induction in others, until the antibody formers are expended (exhaustion). Dorner and Uhr (14) found that prolonged regimens of high antigen doses were necessary to induce a paralyzed state in primed rabbits. However, to discover whether rapid exhaustion of the primed Y cell population occurred before the animal was paralyzed, a study of the critical events during the initial "paralysis induction" is required.

In this report, we have restricted our investigation to one type of unresponsiveness, the exhaustion of memory cells.

To separate Y cell exhaustion from X cell exhaustion, we chose to use the organ fragment culture system developed by Michaelides and Coons (15) as an assay for the content of memory cells. In this system, primed rabbit lymph nodes are induced by antigen challenge to display an entirely in vitro anamnestic response. We could then determine whether putatively exhausting doses of antigen given up to 12 days before in vitro challenge, depleted the memory cell content of primed rabbit popliteal lymph nodes. In this system, a primary response cannot be induced in vitro nor can a primary response induced in vivo be continued in vitro. Furthermore, memory cells which will respond to an in vitro antigenic challenge cannot be generated within 12 days. Hence, any response to the in vitro challenge observed must be due to memory cells which were generated by the original primary injection, 4–6 months earlier.

The experiments described here demonstrate that the memory cell compartment can be productively depleted by a high dose of antigen.

Materials and Methods

Biologicals.—A five-times crystallized bovine serum albumin (BSA) was obtained from Pentex, Inc., Kankakee, Ill. Components of Eagle's medium were purchased from Hyland Laboratories, Los Angeles, Calif. Normal rabbit serum was purchased frozen from Pelfreez Biologicals, Rogers, Ark. Diphtheria toxoid was the gift of Mr. Leo Levine, Massachusetts State Antitoxin Lab, Boston.

Animals and Injections.—For clarity in explication, the first in vivo injection is called the "priming injection," the second is the "booster injection," and the in vitro antigenic stimulus is called the "challenge." New Zealand white rabbits were primed with 10 mg BSA and 100 Lf diphtheria toxoid in each rear foot-pad at an age of about 4 months. 4–6 months later, one animal of a pair was reinjected with a booster of 60 mg BSA in each rear foot-pad and 270 mg BSA intravenously, which was termed an "exhausting" dose. The other was reinjected with a booster of 1 mg BSA in each rear foot-pad and 2 mg intravenously which was termed a "non-exhausting" dose. 4–5 days later, each animal was anesthesized with 100-150 mg Nembutal, administered intravenously, and one popliteal lymph node, which drains the foot-pad, was removed, trimmed of fat, and cultured. Later, the second node was removed from each, cultured, and challenged. The removal of the two popliteal nodes from the same animal at different intervals was designed to minimize biological variation between animals.

Tissue Culture.—The methods and media of Michaelides and Coons (15) were used with slight variations. Eagle's medium was supplemented with 25% normal rabbit serum, 0.1 mM penicillin, 0.04 mM streptomycin, 1 μM hydrocortisone hemisuccinate, and 300 μg/ml L-glutamine. All media were terminally sterilized through Millipore filter membranes. A thin rectangle of extensively washed glass wool, as used by Ambrose (16) was inserted into Leighton tubes, which were then sterilized. The node was cut into cubes about 1 mm^3 and 12 of these fragments were placed in each tube and blanketed with the glass wool. 1 ml medium was added, the tube stoppered, and incubated at 37°C in a 5% CO_2-95% air atmosphere. The media were changed at varying intervals, usually every 2–3 days. In the figures, the titers plotted represent the amount of antibody accumulated in the fluid since the previous entry. The media were titered by passive hemagglutination (17) using a microplate technique (18). Titers are expressed as reciprocals of the $\log_2$ dilution.

When in vitro challenge was required, the fragments were placed in Petri dishes immediately after being cut. They were covered with fresh medium containing 500 μg/ml BSA (unless otherwise stated) and incubated in the gas mixture at 37°C for 2 hr. Care was taken to maintain the pH at exactly 7.4 during this incubation. Each Petri dish was rinsed three times with 10 ml Hanks' balanced salt solution and the fragments were then placed in the flat portion of the Leighton tubes as described above.

RESULTS

The Normal Response.—Fig. 1 shows the typical curve of anti-BSA formation elicited in vitro. Popliteal lymph nodes from rabbits primed 4–6 months earlier were cultured, at which time there was no detectable serum antibody. Some tubes were challenged with 500 μg/ml BSA during the first 2 hr of culture. This graph presents results of five experiments on different animals. The culture medium was changed every 1–3 days, and in each experiment, triplicate tubes or more were used for each condition. The first detectable antibody titer appears on day 4, and reaches a peak on day 9–12, after which it declines. There is a small unchallenged response in some cases, which is observed by many workers in tissue culture systems (19, 20) and is thought to be the result of small amounts of sequestered antigen being released by the culturing procedure. The challenged response is represented in this figure by the solid lines, the unchallenged response found in two of the five animals, by the broken lines.

These titers appear to be lower than those of Michaelides and Coons (15), who found a peak titer of 11 in challenged tubes and five in unchallenged tubes. This actually is a difference in the assay system: when we used the standard macrohemagglutination system in tubes, the titers were always 4–5 $\log_2$ units higher. Despite this fact, we used the microhemagglutination plate procedure for convenience and clarity of end point.

Exhaustion Experiment Protocol.—Two primed animals were given booster injections of either the exhausting or the nonexhausting amount of BSA. 4–5 days later, one popliteal lymph node (the "first node") was surgically removed from each animal, and cultured. At various times thereafter, the contralateral popliteal node (the "second node") was removed from each and cultured with and without in vitro challenge. Changes of tissue culture fluid were made the 1st day after culture, and at 2- or 3-day intervals thereafter.

Production of Antibody after the Exhausting Injection.—Figs. 2 and 3 illustrate the results of two of these experiments. In Fig. 2, the first node was removed 4 days after the booster injection and the titer induced by the in vivo injections can be seen to continue in vitro, in both nonexhausted and exhausted nodes

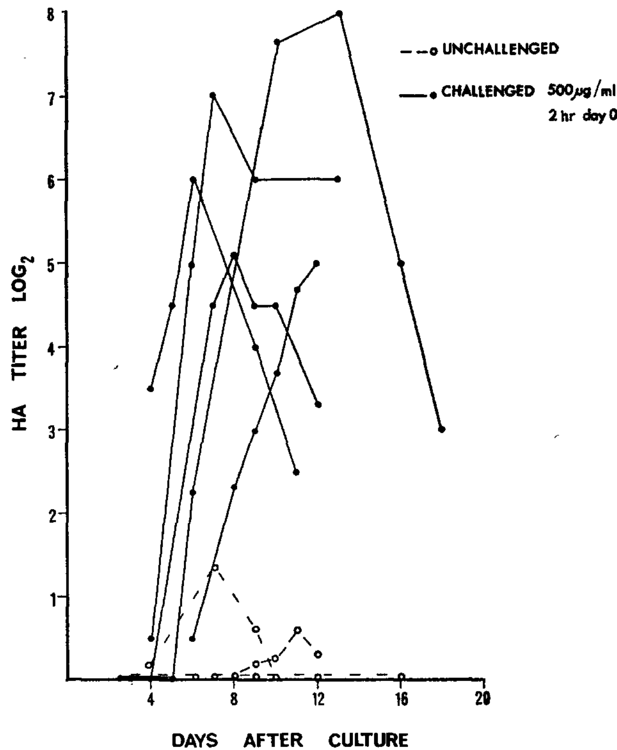


FIG. 1. Typical in vitro responses elicited from fragments taken from rabbit lymph nodes primed 3 months to 1 yr prior to culture. Each point represents the antibody synthesized since the last change of medium, and is the average of three to four tubes. The in vitro challenge was 500 µg/ml BSA for 2 hr on day 0. All titers are expressed as the reciprocal of the $\log_2$ dilution, assayed by hemagglutination.

(Fig. 2, *a* and *b*). The continued response was higher in the animal receiving the exhausting booster than in the one receiving the nonexhausting booster. In both cases, this response rapidly declined to a low level by day 10 or 12. Fig. 3 shows the results of a similar experiment in which the nodes were removed 5 and 12 days after the booster injection. In this pair of animals, although the continuation titers were lower, the exhausted node again displayed the higher titer. It is clear from these results that a vigorous secondary response follows both the exhausting and the nonexhausting booster injection.

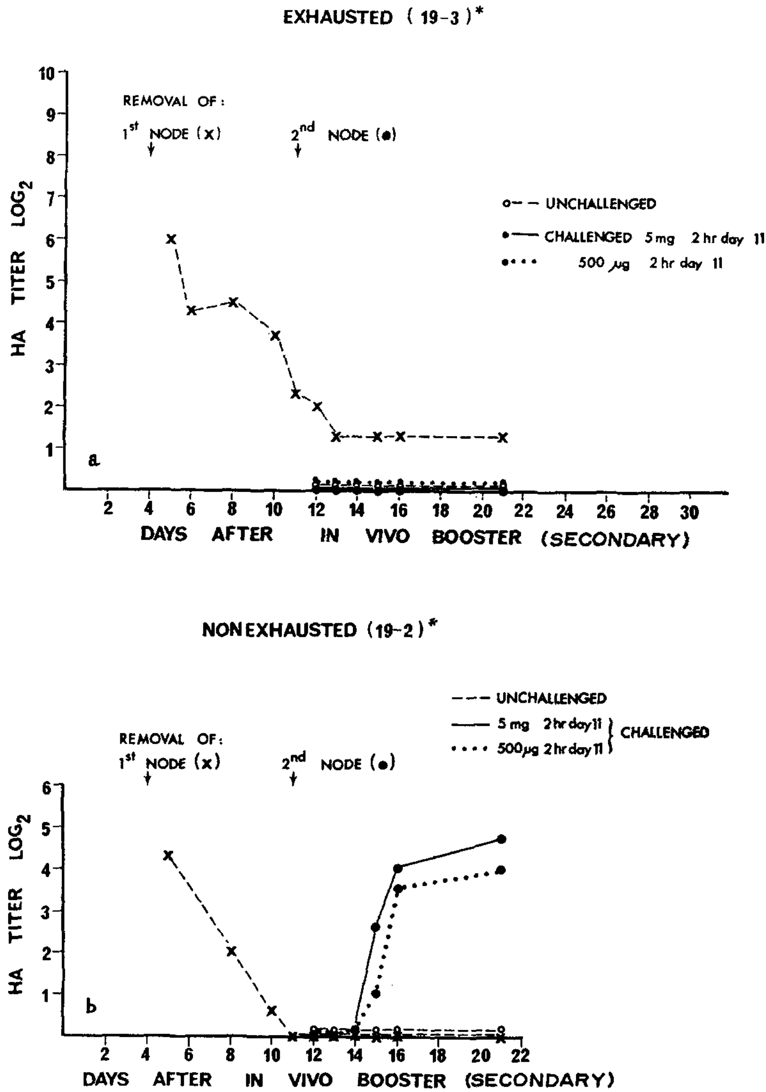


FIG. 2. In vitro response of fragments from (*a*) exhausted or (*b*) nonexhausted rabbit lymph nodes, injected and cultured simultaneously. The first nodes were removed 4 days postbooster and were not challenged. The second nodes were removed 11 days postbooster, and challenged fragments were incubated with 5 mg/ml or 500 μg/ml BSA for 2 hr immediately after preparation. The responses given by the first and second node fragments are shown in one figure. *Individual rabbit number.

Failure to Stimulate Antibody Formation in Exhausted Nodes.—In the experiment shown in Fig. 2, the second node was removed on day 11, at which time the titer of the first node cultures was quite low. The node fragments in some of the tubes were challenged with 500 μg/ml BSA in vitro, and those in other

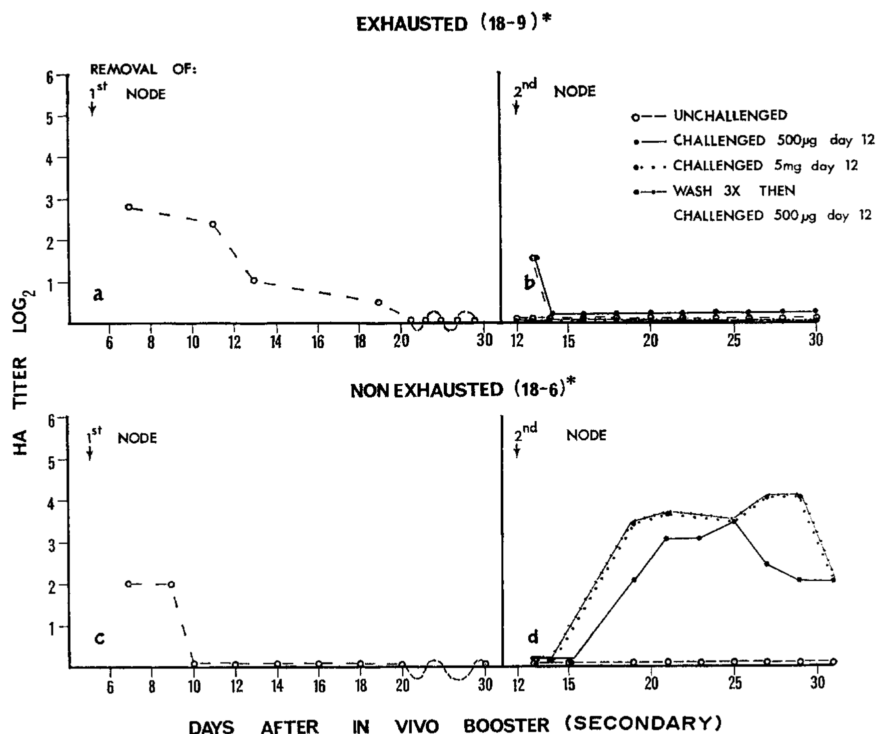


FIG. 3. In vitro response of fragments from (*a*) exhausted or (*c*) nonexhausted first nodes removed 5 days postbooster and not challenged, and fragments from exhausted (*b*) or nonexhausted (*d*) second nodes removed 12 days postbooster. These latter fragments were challenged with 500 μg/ml BSA or 5 mg/ml BSA for 2 hr immediately after preparation, or washed three times and then challenged with the two BSA concentrations for 2 hr; or not challenged.

tubes received no challenge. The 1st day after culture, neither the exhausted nor the nonexhausted nodes were producing a detectable level of antibody; however by the 4th day (15 days postbooster), the challenged, nonexhausted nodes (Fig. 2, *b*) had begun their anti-BSA response, which rose to a peak 1 wk after culture, in a mode analogous to the typical in vitro secondary response shown in Fig. 1. It should be noted that the titers produced by these challenged, nonexhausted fragments are lower than those in Fig. 1. This is to be expected, since a portion of the Y cell population generated by the primary in vivo injec-

tion was expended by the booster injection. In contrast, although the exhausted fragments also produced a secondary response in vivo to the booster injection, they were subsequently incapable of being restimulated to produce antibody in vitro. Fig. 3, *b* and *d* show a similar pattern; although the nonexhausted node fragments could respond to challenge with antibody production which peaked on days 9 through 11, the exhausted node was refractory to challenge.

Failure of the Booster Injection to Induce an In Vitro Response.—To prove that the challenged response elicited from nonexhausted nodes was due to residual memory cells generated by the priming injection, and not to memory cells generated by the low booster injection, unprimed animals were injected with exhausting or nonexhausting doses, and the nodes were cultured at intervals of 4, 7, and 12 days afterward. Titers were negative in both challenged and unchallenged tubes, demonstrating that within the time period used, a primary response could not be continued in vitro, nor were sufficient memory cells generated in vivo to permit successful in vitro challenge.

Possible Explanations for the Failure to Restimulate Exhausted Nodes.—Our tentative explanation for the failure to restimulate a response by the in vitro challenge rests on the assumption that the population of Y cells was functionally absent in exhausted nodes. This may have resulted from their productive expenditure in one-way differentiation to plasmacytic, terminal Z cells. On the other hand, some Y cells may have been present which were not capable of responding to challenge.

We tried to explore several alternatives to exhaustion. One possibility was that antibody produced by the exhausted node was present in the medium during the 2 hr in vitro challenge, complexing with the challenge BSA at ratios too rich in antibody to provide an effective stimulus. Since several workers have shown that complexes at equivalence or in antigen excess can stimulate a good response (21, 22), a 10-fold higher level of challenge BSA was used to assure the presence of a zone of antigen excess. Thus, we attempted 2-hr stimulation with 5 mg/ml BSA, with the results shown in Fig. 2, *a* and *b*, and Fig. 3, *b–d*. Although the nonexhausted nodes responded to this treatment with slightly higher titers of antibody, the exhausted fragments remained unresponsive.

A further possibility was that the exhausted node might have made some substance, possibly a variety of antibody with particular feedback effectiveness, which could interact with memory cells and inhibit induction of further antibody responses. Although unlikely, on the grounds that the nonexhausted nodes were also very recently guilty of antibody production, this was tested in two ways. (*a*) The fragments were washed three times with an excess of Hanks' solution prior to challenge. In the nonexhausted node, this resulted (Fig. 3 *b–d*) in a higher titer comparable with that given in response to the higher antigen dose of 5 mg/ml mentioned previously. However, although the washing removed the small residual antibody which would have dissociated from the exhausted

fragments during the 1st day of culture, they were still unresponsive to challenge. Hence it is concluded that although there may be a small amount of antibody bound to the cell surface in both exhausted and nonexhausted nodes, it is dissociable and cannot account for their incapacity to respond. (*b*) A mixture of exhausted and nonexhausted fragments were incubated for 3 hr before in vitro challenge and culture. Each Leighton tube contained about six non-

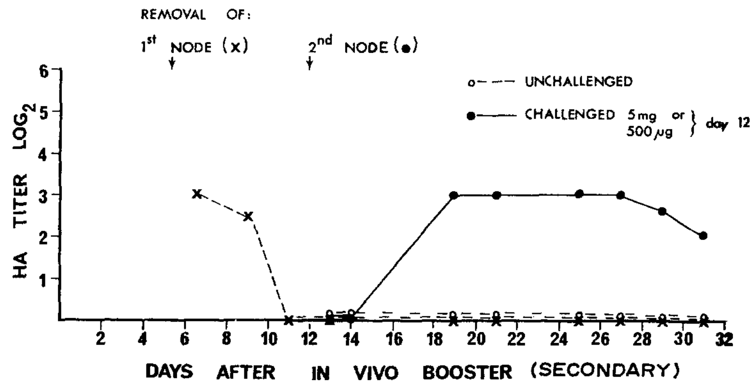


FIG. 4. In vitro response obtained from a mixture of the exhausted (rabbit 18-9) and nonexhausted (rabbit 18-6) node fragments shown in Fig. 3. The responses of the first and second node fragments are shown in the same figure.

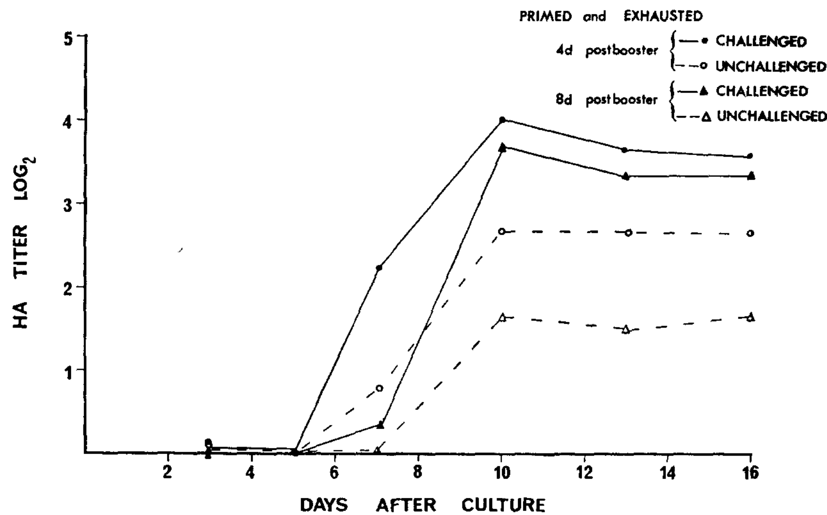


FIG. 5. In vitro response of an equal mixture of fragments from a node primed 1 yr before culture with fragments from an exhausted node removed either 4 or 8 days postbooster. The standard challenge was performed.

exhausted node fragments and six exhausted node fragments. Fig. 4 shows the results. The mixture of nodes removed 5 days postbooster, during the in vitro continuation phase of the response, showed titers about half as high as those seen in tubes containing 12 exhausted fragments cultured alone. The mixture of node fragments removed 12 days postbooster responded to challenge with titers about half as high as those seen in tubes containing the nonexhausted node fragments cultured alone, as if only half of the fragments (presumably the nonexhausted ones) were responding.

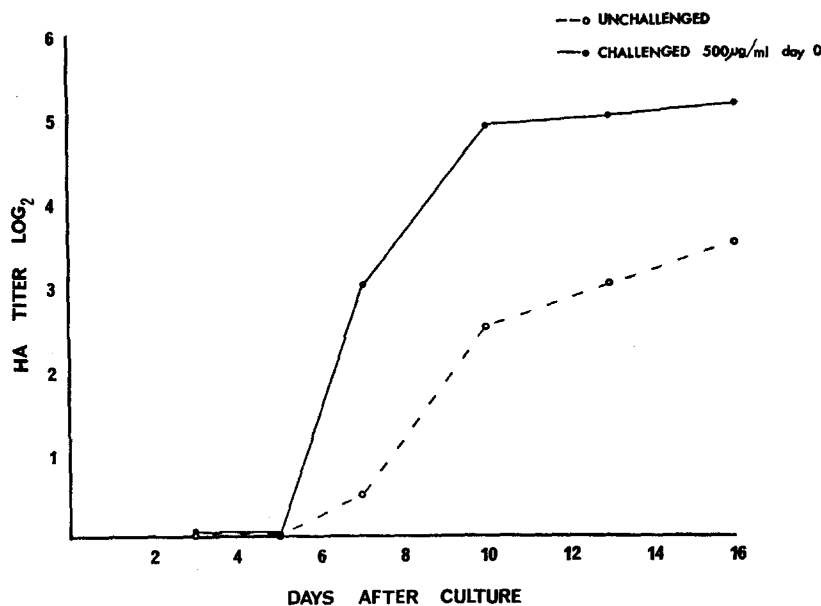


FIG. 6. In vitro response of the primed node (rabbit 17-3) used in Fig. 5, when challenged and cultured alone. When the exhausted nodes were cultured alone, they were refractory to challenge.

In addition, exhausted nodes were removed at intervals of 4 and 8 days after the booster injection and mixed with node fragments obtained from an animal primed 1 yr earlier. The two node pieces were cut together in the same small Petri dish in a volume of 1 ml of medium. This dish was then incubated, 37°C for 3 hr, without washing or further addition of medium. After one wash, the fragments were challenged as usual. Although each of the exhausted nodes were producing small quantities of antibodies at these times, the mixture could still be challenged to produce a response (Fig. 5) slightly lower than that given by the primed fragments alone (Fig. 6). As usual, the exhausted nodes were refractory when cultured alone.

Two conclusions may be drawn from these experiments. (*a*) A response can be induced by an in vitro challenge of 500 μg/ml BSA even in the presence of active antibody production by an exhausted node. (*b*) There is no substance, antibody or other, produced by the exhausted node fragments 4, 8, or 12 days postbooster which interacts with memory cells and prevents stimulation.

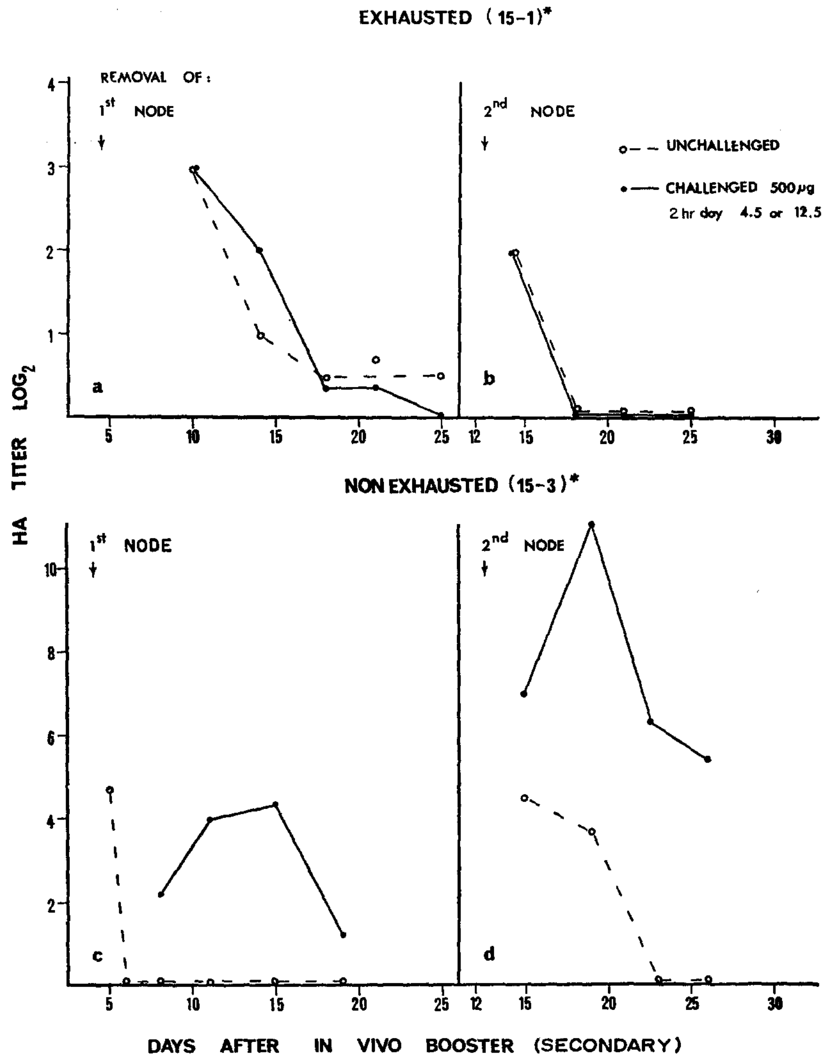


FIG. 7. In vitro challenge of (*a*) exhausted or (*c*) nonexhausted first node fragments, cultured and challenged 4.5 days postbooster, and (*b*) exhausted or (*d*) nonexhausted second node fragments, cultured and challenged 12.5 days postbooster.

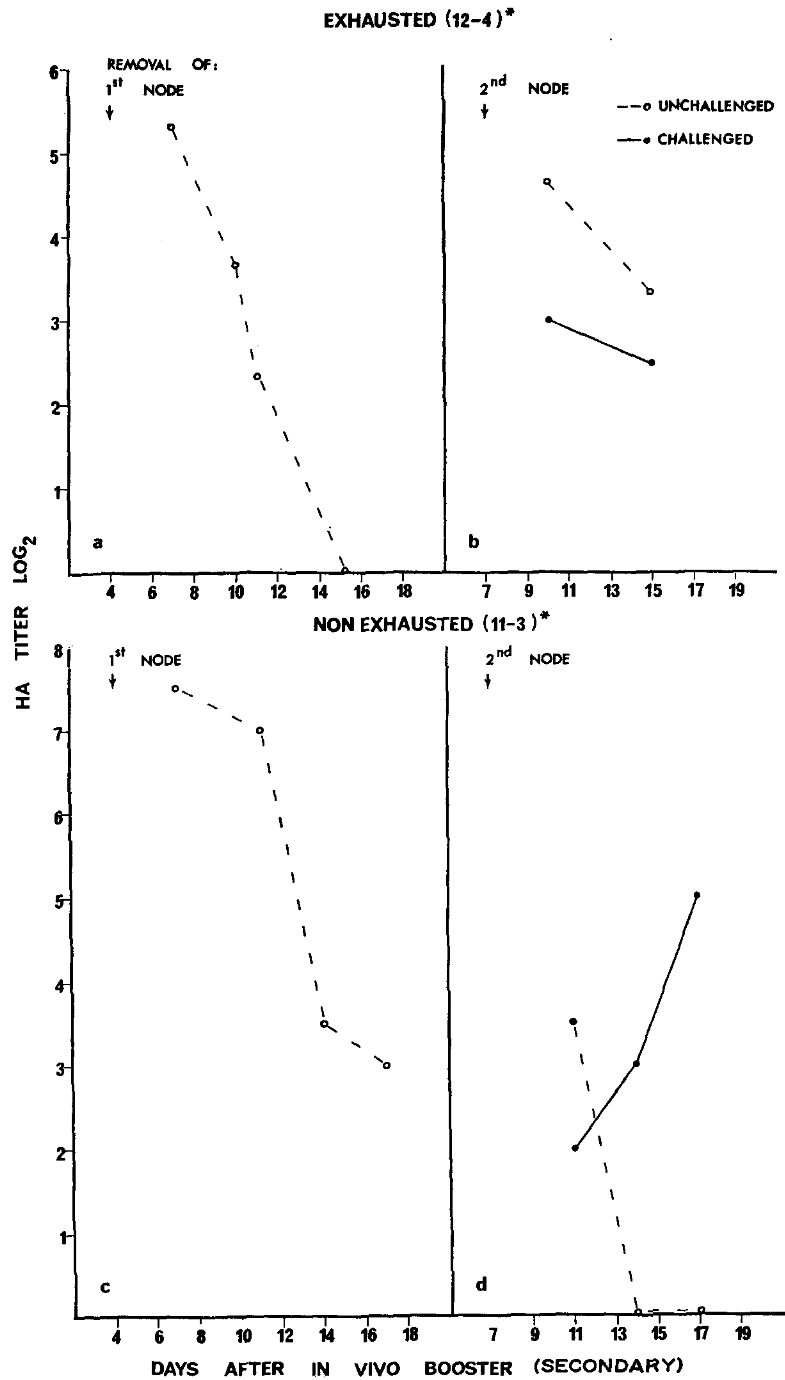


FIG. 8. In vitro challenge of second nodes removed and challenged 7 days postbooster (*b* and *d*). Continuation of the response given by the first node, removed 4 days postbooster shown in frames (*a*) and (*c*).

Additional pairs of exhausted and nonexhausted cultures are shown in Figs. 7 and 8. They again demonstrate that nonexhausted nodes can respond to challenge even in the presence of active antibody production.

"First node" stimulation.—Nonexhausted nodes have been cultured and challenged at intervals of 4, 5, 7, 11 and 12 days after the booster injection. The nonexhausted nodes removed 7 days or more postbooster were capable of responding to challenge. However, only two out of four nonexhausted nodes removed on day 4 or 5 postbooster were capable of responding to this antigenic challenge, even when the level was increased to 5 mg/ml BSA. The reason for this incapacity is unknown.

Concomitant X Cell Paralysis.—It is probable that the exhausting booster injection not only exhausts the memory cells, but also temporarily paralyzes the X cell population. We attempted to determine whether the exhausting booster would permit some X→Y conversion if sufficient time were allowed to elapse before culture. Even 4 wk after the exhausting booster, the nodes were still refractory to stimulation. Therefore, it seems that the high exhausting booster dose does not allow memory cell reappearance in the node for at least 1 month.

DISCUSSION

The concept of exhaustion of precursors of antibody-producing cells is not new. Taliaferro and Taliaferro showed in 1951 that multiple antigen doses reduced the response of the spleen to further challenge (7). This temporary refractoriness could not be explained by the neutralization of antibody by antigen. In spleen transfer experiments, they showed that the refractoriness was a cellular attribute (8, 23). Other adult injection regimens in diverse systems have led to states of temporary unresponsiveness (e.g. 24–26). However, without further investigation, it would be tenuous to assume that these are examples of exhaustion, for there are several alternatives. The unresponsiveness might be due to paralysis, or it might involve feedback effects. Even if these two causes are eliminated, and a particular case is shown to have the characteristics of exhaustion, i.e. antibody formation followed by a proven cellular inability to respond to antigenic stimulus, experimental models have rarely attempted to distinguish between exhaustion at the level of virgin cells (X) and exhaustion at the level of memory cells (Y). For example, Šterzl showed that injecting neonatal rabbits with large doses of *Salmonella paratyphi B* resulted in high initial antibody titers, but unusually low secondary titers (27). He also used localized hemolysis in gel to study the number of cells producing anti-sheep red cell antibody, and showed that the number of active cells in the secondary reaction were in inverse proportion to the size of the primary antigenic stimulus (28). Hege and Cole reported that after injecting mice with a single dose of 4×10^8 sheep red cells, 9 wk must elapse before a

booster injection would give a response of 19S plaque-forming cell numbers equivalent to those of the primary response (29). These are probably examples of exhaustion involving both X cells and Y cells; however, antibody feedback effects are difficult to rule out.

In this paper, it has been shown that the memory cell population of a primed rabbit lymph node may be exhausted by a large booster dose. This results in an initial burst of antibody formation, followed by a refractory period during which the node is functionally deprived of memory. In order to classify this type of unresponsiveness as Y cell exhaustion, (*a*) it must be distinguished from paralysis in which no detectable antibody is initially produced, and (*b*) it must be shown to exist on the level of the memory cell.

In designing an experiment to meet these two criteria, we reasoned as follows: if a primed rabbit were given a supraoptimal dose of antigen, the draining lymph node, cultured 4–5 days postbooster should show an in vitro continuation of the booster response, which would satisfy the first criterion. If the memory cell population had been exhausted by this large booster injection, the contralateral draining node, removed before enough time had elapsed for new memory cells to have been recruited by the booster injection, should then be incapable of responding to an in vitro challenge. In contrast, a similar primed rabbit, given a nonexhausting booster injection should also demonstrate the same in vitro continuation of the response; however, its node should still be capable of responding to a third in vitro challenge, since only part of the population of Y cells would have been triggered by this booster injection. This tertiary response should have the same kinetics as a typical secondary response induced in vitro, although the peak titer would probably be lower, since part of the memory cell population would have been expended during the booster response.

Rabbits were given primary injections of BSA in the rear foot-pads, and after the primary serum antibody had disappeared they were given either exhausting or nonexhausting booster injections. The draining nodes were cultured with or without challenge as described above. The results followed our expectations. Both exhausted and nonexhausted nodes could be seen to continue their booster response in vitro. After this tapered off, the exhausted node fragments were subsequently refractory to in vitro antigenic challenge, while the nonexhausted node fragments could respond to this challenge.

To meet the second criterion and show that the tertiary response given by the nonexhausted node fragments was due only to residual Y cells which had been generated at the time of the primary injection, 4–6 months earlier, it was necessary to demonstrate that the booster injection neither induced a primary response nor generated memory cells which could be detected in this system. Accordingly, it was shown that in neither normal nodes, nor those removed 4, 7, or 12 days after a primary injection of either antigen dose could a response be induced or continued in vitro. Evidently some cellular or chemical

mediation, present in vivo and lacking in vitro is necessary for the continuation of the primary response and the generation of memory cells. To our knowledge no investigator has ever continued a primary response in vitro with BSA. Vischer and Stastny, using a system similar to ours, could continue a primary response to hemocyanin in vitro, but not that to BSA (30).

The inhibitory effect of specific antibody on further antibody production has been well documented in sheep red cell systems (31–33), antiphage systems (34), and protein antigen systems (35). However it seemed doubtful that antibody feedback was responsible for the refractoriness of these exhausted nodes for two reasons. (*a*) Both exhausted and nonexhausted nodes had produced significant amounts of antibody in response to the booster injection. If antibody within the exhausted node was preventing the antigen from effectively reaching its target, the same problem should be encountered in the nonexhausted nodes. This was not the case; furthermore, we showed that in vitro stimulation can occur in the presence of antibody. (*b*) The work of Rowley and Fitch has shown that antibody feedback operates primarily on the level of "potential antibody producers" (X cells) and specific antibody only slightly lowers the responsiveness of "antibody-producing cells" (Y cells) (33). Others have found similar results.[2,3]

Nevertheless, we experimentally explored the role of antibody feedback in our system. Two possibilities were considered: that ambient antibody was preventing the challenge antigen from reaching its target memory cells, or that the exhausted node was secreting a substance (feedback antibody) during its booster response, which prevented expression of untriggered memory cells. These experiments, documented in the Results section, all lead to the conclusion that if the memory cell is present we can stimulate it. This renders feedback explanations unlikely.

In experiments designed to demonstrate the transience of Y cell exhaustion, nodes were removed as late as 4 wk after the exhausting booster injection, since it was assumed that sufficient X→Y conversions would have occurred by this time to allow a stimulated response in vitro. The fragments were still refractory. Therefore, it was concluded that the exhausting booster prevented in vivo X→Y conversions, and that in this case, Y cell exhaustion was probably coupled with X cell paralysis.

If exhaustion of memory is true, and not a complicated artifact, it is necessary to give a cellular explanation for memory retention. Several hypotheses are illustrated in Fig. 9. The first suggests that the virgin X cell is induced by contact with antigen to undergo transformation to a Y (memory) cell. Following a second antigenic stimulus, the cell would begin symmetrical division and maturation to a clone of terminal plasmacytes, called Z_s cells (Fig. 9, 1). This

[2] Dixon, F. J. Personal communication.

[3] Uhr, J. W., and G. Möller. Review to be published.

second antigenic stimulus would also induce new $X \rightarrow Y$ conversions in the animal, which would maintain the memory. Repeated injections of antigen might eventually deplete the animal of X cells.

A second alternative is presented in Fig. 9, 2. Some or all of the memory cells respond to the second antigenic stimulus by dividing asymmetrically to produce one memory cell and one Z_a cell, which continues its further differentiation to a Z_z cell. In this way there would be a retention of memory cells.

A third group of theories holds that a memory cell differentiates into plasma cells, some small percentage of which retain the capacity for responding to further stimulation by one of two mechanisms, which we have termed "resuscitation," and "rejuvenation." These are diagrammed in Fig. 9, 3. Both of these mechanisms have in common the fact that a terminal or near terminal cell serves as the basis for memory retention. Resuscitation may be conceived of as the reactivation of cells temporarily halted along the pathway of $Y \rightarrow Z_z$ maturation by some regulatory process. Antigen, presumably, is the resuscitating agent. Two forms of resuscitation are indicated by bold arrows in the forward direction: one in which a Z_z cell survives as an antigen-sensitive cell (36), and one in which a special Z_y cell survives as a late antigen-sensitive, asymmetric offshoot of the usual pathway. Rejuvenation is thought of as a recycling of late cells to an earlier stage in maturation, probably accompanied by a change in appearance, but not a change in specificity. Two rejuvenations are shown,the first to a typical Y cell, and a second to a type of cell which may be termed Z_g to signify an IgG memory cell at a maturation level beyond the formation of IgM antibody.

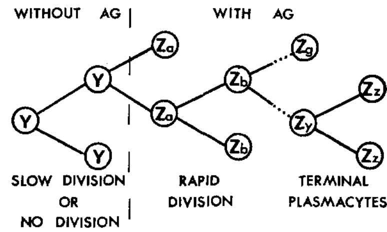
We feel that these experimental results are consistent with the first mechanism pictured, but not with the second, where asymmetric division of Y cells is the general mode of memory retention. It is conceivable that the asymmetrically produced memory cell is quite motile, and escapes into the circulation shortly after formation. It would therefore not be present in the cultured node when the antigenic challenge is applied. However, the rat thoracic duct drainage experiments of McGregor and Gowans indicated that no appreciable part of the memory cell population recirculates (37). This complements the evidence of Sercarz and Coons regarding the temporary refractoriness of sessile lymphoid tissue following an exhausting dose of antigen (3), and supports the conclusion that in exhausted animals, there is indeed an absence of memory cells which can be stimulated.

Our experiment does not rule out any of the various forms of rejuvenation, but only places a time limit on them; if rejuvenations do occur in any appreciable number, the process requires a time period greater than 4 wk.

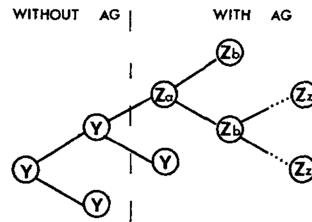
However, the resuscitation theories are more difficult to reconcile with the results reported here. If this is a major form of memory retention, it must be stringently limited to cases in which the maturation halt is caused by an entirely intracellular controlling agent which persists for longer than 4 wk, has

no effect on surrounding cells, and which is produced by only a small fraction of memory cell progeny. This seems unlikely because of experiments indicating that there is suicidal differentiation of memory cells upon antigenic stimulation and destruction of the resultant mature plasmacytes (38). If resuscitation

1. SYMMETRIC DIVISION OF Y CELLS



2. ASYMMETRIC DIVISION OF Y CELLS



3. TERMINAL CELLS AS MEMORY CELLS

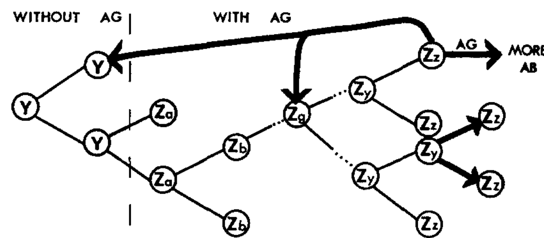


FIG. 9. Theories of origin and fate of memory cells. For ease of presentation, only one of the progeny cells from any division is followed. There is meant to be a continuous progression from Z_a, the first Z generation, to Z_z, the last Z generation. No implication about the exact number of cell generations is implied by the symbolic subscripts.

is a mechanism of memory retention, it must be limited to a few hardy survivors, below our threshold of detection.

SUMMARY

A set of conditions has been described under which primed rabbit lymph nodes produce a secondary antibody response upon in vivo stimulation with

a large dose of antigen, but are subsequently "exhausted;" that is, lymph node cultures prepared at intervals following the booster injection cannot be restimulated to display tertiary responses. Rabbits given 100-fold less antigen in the booster inoculum were able to give a tertiary response upon in vitro challenge. The system used permits neither induction nor continuation of a primary response to BSA in vitro. Since it could be demonstrated that no memory cells were generated by the booster injection within the intervals between in vivo injection and culture, the tertiary response in nonexhausted nodes must have been due to residual memory cells which remained untriggered by the in vivo booster injection.

The unresponsive state was not caused by antibody feedback.

These results are interpreted to mean that a population of memory cells can be exhausted by a supraoptimal dose of antigen, rendering the node temporarily incapable of further response. This implies that long-lived memory is not due to asymmetric division of memory cells. The source and fate of memory cells is discussed with regard to this evidence.

We would like to thank Anne Clark for her ebullient and expert technical assistance. This research was supported in part, by Grants GB-1619 and GB-5410 from the National Science Foundation, and Grant ACS-E-4S4 from the American Cancer Society.

BIBLIOGRAPHY

1. Burnet, F. M., and F. Fenner. 1949. The Production of Antibodies. Macmillan and Company, London. 2nd edition.
2. 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. Exptl. Med.* **102**:61.
3. Sercarz, E. and A. H. Coons. 1962. The exhaustion of specific antibody producing capacity during a secondary response. *In* Mechanisms of Immunological Tolerance. M. Hašek, A. Lengerova, and M. Vojtiskova, editors. Czechoslovak Academy of Sciences Press, Prague. 73–83.
4. Šterzl, J., and A. M. Silverstein. 1967. Developmental aspects of immunity. *Advan. Immunol.* **6**:337.
5. Hašek, M., A. Lengerova, and T. Hraba. 1961. Transplantation immunity and tolerance. *Advan. Immunol.* **1**:1.
6. Smith, R. T. 1961. Immunological tolerance of non-living antigens. *Advan. Immunol.* **1**:67.
7. Taliaferro, W. H., and L. G. Taliaferro. 1951. The role of the spleen in hemolysin production in rabbits receiving multiple injections. *J. Infect. Diseases.* **89**:143.
8. Taliaferro, W. H. 1955. Studies in antibody formation. *In* Some Biological Aspects and Consequences of Parasitism. W. H. Cole, editor. Rutgers University Press. New Brunswick. 50.
9. Eisen, H. N. 1951. Precipitin reaction between hapten-azoprotein and anti-hapten rabbit sera. *Federation. Proc.* **10**:179.
10. Talmage, D. W., G. G. Freter, and W. H. Taliaferro. 1956. Effect of repeated

injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums. *J. Infect. Diseases.* **98**:293.

11. Sercarz, E. and A. H. Coons. 1960. The exhaustion of specific antibody-producing capacity during a secondary antibody response. *Federation. Proc.* **19**:199.
12. Dresser, D. W. 1962. Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. *Immunology.* **5**: 378.
13. Golub, E. S. and W. O. Weigle. 1967. Studies on the kinetics of immunologic unresponsiveness in adult mice. *Federation Proc.* **26**:417.
14. Dorner, M. and J. Uhr. 1964. Immunologic tolerance after specific immunization. *J. Exptl. Med.* **120**:435.
15. Michaelides, M. and A. H. Coons. 1963. Studies on antibody production. V. The secondary response in vitro. *J. Exptl. Med.* **117**:1035.
16. Ambrose, C. T. 1964. The requirements for hydrocortisone in antibody-forming tissue cultivated in serum-free medium. *J. Exptl. Med.* **119**:1027.
17. Boyden, S. V. 1951. The absorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera. *J. Exptl. Med.* **93**:107.
18. Sever, J. L. 1962. Applications of a microtechnique to viral serological investigations. *J. Immunol.* **88**:320.
19. Halliday, W. J. and J. S. Garvey. 1964. Some factors affecting the secondary immune response in tissue cultures containing hydrocortisone. *J. Immunol.* **93**: 757.
20. Tao, T. W. 1964. Phytohemagglutinin elicitation of specific anamnestic immune response in vitro. *Science.* **146**:247.
21. Terres, G. and S. L. Morrison. 1967. Enhanced immunologic sensitization of mice by the simultaneous injection of antigen and specific antiserum. III. The role of antigen in controlling the immune response elicited with immune complexes. *J. Immunol.* **98**:584.
22. Leskowitz, S. 1960. Studies in immunization. II. Immunization with specific precipitates. *J. Immunol.* **85**:56.
23. Taliaferro, W. H. and L. G. Taliaferro. 1954. Transfer of antibody-forming capacity in splenic materials. *Science.* **119**:585.
24. Dixon, F. J. and P. H. Maurer. 1955. Immunologic unresponsiveness induced by protein antigens. *J. Exptl. Med.* **101**:245.
25. Rowley, D. A. and F. W. Fitch. 1965. I. Plaque-forming cell and antibody response to single and multiple injections of antigen. *J. Exptl. Med.* **121**:671.
26. Mitchison, N. A. 1964. Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. London, Ser. B.* **161**:275.
27. Šterzl, J. and Z. Trnka. 1957. Effect of very large doses of bacterial antigen on antibody production in new born rabbits. *Nature.* **179**:918.
28. Šterzl, J. 1966. Immunological tolerance as a result of terminal differentiation of immunologically competent cells. *Nature.* **209**:416.
29. Hege, J. S. and L. S. Cole. 1966. Antibody plaque forming cells: kinetics of primary and secondary responses. *J. Immunol.* **96**:559.
30. 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.
31. Wigzell, H. 1966. Antibody synthesis at the cellular level. Antibody induced suppression of 7S antibody response. *J. Exptl. Med.* **124**:953.
32. Möller, G. and H. Wigzell. 1965. Antibody synthesis at the cellular level. Antibody-induced suppression of 19S and 7S antibody response. *J. Exptl. Med.* **121**: 969.
33. Rowley, D. A. and F. W. Fitch. 1964. Homeostasis of antibody formation in the adult rat. *J. Exptl. Med.* **120**:987.
34. Uhr, J. W. 1964. The heterogeneity of the immune response. *Science.* **145**:457.
35. Dixon, F. J., H. Jacot-Guillarmod, and P. J. McConahey. 1967. The effect of passively administered antibody on antibody synthesis. *J. Exptl. Med.* **125**:1119.
36. Miller, J. J., III. 1964. An autoradiographic study of plasma cell and lymphocyte survival in rat popliteal lymph nodes. *J. Immunology.* **92**:673.
37. McGregor, D. D. and J. L. Gowans. 1963. The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct. *J. Exptl. Med.* **117**:303.
38. Makinodan, T. and J. F. Albright. 1967. Proliferative and differentiative manifestations of cellular immune potential. *Progr. Allergy.* **10**:1.