

IN VITRO-INITIATED SECONDARY ANTI-HAPTEN RESPONSE

II. INCREASING CELL AVIDITY FOR ANTIGEN*

BY WESLEY W. BULLOCK AND MARVIN B. RITTENBERG,† PH.D.

(From the Department of Microbiology and the Division of Immunology and Allergy,
University of Oregon Medical School, Portland, Oregon 97201)

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The hypothesis that antigen causes population shifts in responding immunocompetent cells through competition among these cells for antigen (1) is in accord with the following experimental observations. Antibodies with increased "avidity" for antigen appear following immunization (2). Ligand affinity of anti-hapten antibody can rise several orders of magnitude in serum samples taken a few weeks apart (3). This increase results from synthesis of new molecules with greater affinity for the hapten (4). High affinity antibody appears earlier when smaller antigen doses are used for immunization (4). Similarly thymidine incorporation *in vitro* indicates that animals sensitized by low antigen doses yield cells more responsive to low dose challenge than do animals sensitized by high doses (5). The ability to induce an anamnestic anti-hapten response with hapten-heterologous carrier conjugates increases with time after sensitization *in vivo* (6-9) and correlates with the appearance of high affinity anti-hapten antibody (8-9). The above evidence, largely contributed by studies of antibody affinity, suggests that antigen brings about a gradual shift in the specific immunocompetent population until late in immunization when it consists mainly of cells bearing high affinity receptors and synthesizing high affinity antibody (10). This hypothesis assumes that the affinity of a particular antibody for its specific ligand reflects the affinity of antigen receptors on the cell which synthesized the antibody. However, few attempts have been made to measure directly the cellular changes in immunocompetent populations. If, as suggested above, antigen preferentially selects responsive cell populations with surface receptors of higher affinity, then later in the immune response less antigen should be required to stimulate these newly-arising cells. Systematic studies of this predicted increase in cellular sensitivity to antigen have not yet been described.

We reported previously that trinitrophenyl-hemocyanin (TNP-KLH)¹ initiates a

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† Scholar of the Leukemia Society of America.

¹ *Abbreviations used in this paper:* BSA, Bovine serum albumin; KLH, hemocyanin; N-SRBC, nonhaptentated SRBC; PFC, plaque-forming cells; SRBC, sheep red blood cells; T-K-B, TNP-KLH coated onto bentonite; TNP, trinitrophenyl; TNP-SRBC, trinitrophenyl-haptentated sheep red blood cells.

vigorous secondary anti-hapten response in vitro (11) as detected by anti-hapten plaque assay (12). This system offers two main advantages for detecting cell populations with increased antigen sensitivity. (a) Stimulating the anamnestic response in vitro insures exposure of the entire immunocompetent population to a known concentration of antigen enabling a comparison of dose effects. (b) Detecting only cells synthesizing anti-TNP avoids variability resulting from additive responses to multiple, uncharacterized determinants on complex immunogens.

Our results indicate that anti-hapten memory cells with increased sensitivity for antigen in vitro arise in vivo following immunization of BALB/c mice with TNP-KLH. During a 1-4 month resting period this sensitivity to antigenic stimulation in vitro may increase 1000-fold. This result supports the hypothesis that competition for antigen selects memory cells with high-affinity antigen receptors.

Materials and Methods

Mice.—Adult BALB/c female mice were obtained from Simonsen Laboratories, Inc., Gilroy, Calif. They were caged in groups of eight with free access to food and water. The mice were first immunized when 2-5 months old.

Proteins.—Hemocyanin (KLH) was obtained from keyhole limpets (*Megathura crenulata*) by the method of Campbell, et al. (13). TNP-KLH was prepared as described previously (14). Mole ratios of TNP:KLH ranged from TNP₈₅₀KLH to TNP₁₀₀₀KLH. The particulate form of antigen was prepared by coating TNP-KLH onto bentonite (T-K-B) as described previously (12). Trinitrophenyl-bovine serum albumin (TNP-BSA) was prepared as in reference 14. It had a TNP:BSA mole ratio of TNP₁₅BSA.

Spectrophotometric determinations of hapten:protein ratios were performed as in reference 14. Protein was determined by nesslerization (13).

Immunization.—Mice were primed for later secondary in vitro stimulation by intraperitoneal injections of T-K-B every 2 wk for a total of three injections. Each injection contained 100 μ g protein in 0.5 ml saline.

Cell Culture.—Mice were rested 1-4 months after priming, killed by cervical dislocation, and their dissociated spleen cells cultured to detect antigen-sensitive memory cells. To minimize individual variation between mice, spleen cells from at least three mice were pooled in each experiment. Cells were cultured and harvested for plaque assay by the method of Mishell and Dutton (15) as described earlier (11). Each culture contained $1-1.5 \times 10^7$ nucleated spleen cells. Cells were counted on a Coulter Counter. Culture medium contained 10% fetal calf serum ("Rehatuin" E21505 Reheis Co., Inc., Berkeley Heights, N. J.) and 50 Units/ml each of penicillin and streptomycin (Microbiological Associates, Inc., Bethesda, Md.). Experimental groups cultured in vitro received various concentrations of soluble TNP-KLH antigen or particulate T-K-B antigen as indicated in Results. We reported previously that similar protein concentrations of soluble or particulate TNP-KLH induce comparable secondary responses in vitro (11). Control groups did not receive antigen. Antigen was added at initiation of culture (day 0).

Detection of In Vitro Anti-TNP Response: Anti-TNP Plaque Assay.—TNP-haptenated sheep red blood cells (TNP-SRBC) were prepared according to the method of Rittenberg and Pratt (12). Cultured cells were scraped from the culture dishes and packed by centrifugation (11); culture supernatants were removed for separate analysis. Cells were resuspended in appropriate volumes (1-4 ml) of cold Eagle's minimal essential medium, counted, and plated in quadruplicate with TNP-SRBC and with nonhaptenated SRBC (N-SRBC). The responses

are reported as anti-TNP plaque-forming cells (PFC)/ 10^6 cells assayed. A low background plaque count was obtained with N-SRBC (<18 PFC/ 10^6).

Cultures were tested for cells producing IgM and IgG antibodies (11). Cells producing IgM anti-TNP antibody were detected by direct plating. Cells producing IgG anti-TNP antibody were detected by adding rabbit or goat anti-mouse globulin antiserum with the complement. Our facilitation serum inhibits 19S PFC approximately 25–30%; therefore, total facilitated plaques were taken as 7S PFC (12, 16). Guinea pig serum diluted 1:20 was used for complement. Guinea pig serum and anti-globulin serum were absorbed with SRBC prior to use (12). The anti-globulin serum was heated at 56°C for 30 min prior to use.

Supernatant Titration.—The lytic spot test of Hübner and Gengozian (17) was modified to titer small amounts of anti-TNP antibody in culture supernatants. Soft agar layers containing 0.47% TNP-SRBC or N-SRBC were used. Drops of diluted supernatant were placed on these layers, allowed to soak in, and incubated for 1 hr at 37°C, after which the plates were developed as in the anti-TNP plaque assay. Supernatant samples were treated with 0.1 M 2-mercaptoethanol for 30 min at 37°C, dialyzed against 0.01 M iodoacetamide, and then against 0.85% saline (18). Lytic spot testing was then used to determine the presence of 19S and 7S antibody in the supernatant.

^{14}C Amino Acid Incorporation.—Cultures were tested for protein synthesis by adding 1 μCi each of ^{14}C isoleucine and ^{14}C valine to cultures on day 4. Supernatants were concentrated by incubation with polyacrylamide gel (Lyphogel, LKB Instruments, Inc., Rockville, Md.). ^{14}C amino acid incorporation in total supernatant protein was estimated by the method of Timourian and Denny as described in reference 19. The supernatants were dialyzed against three changes of buffered saline prior to adsorption on chromatography paper strips. The protein was precipitated on the strips with 10% trichloroacetic acid. Duplicate samples were prepared and the counts averaged after subtraction of background. Background was approximately 1% of the total counts. Incorporation of ^{14}C -amino acids into specific anti-TNP was determined by adding TNP-BSA and anti-BSA to sixfold concentrated culture supernatants. Antigen-antibody precipitates were allowed to form at 37°C for 1 hr followed by 4°C for 3 days. The precipitates were then washed twice in cold saline, digested in NCS (Nuclear-Chicago Corp., Des Plaines, Ill.), and counted in a Beckman Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, Calif.).

Radioimmuno-electrophoresis.—The method of Thorbecke and Hochwald (20) was used. Culture supernatants were concentrated threefold and electrophoresed with unlabeled mouse serum containing anti-TNP antibody. Following electrophoresis TNP-BSA, TNP-KLH, BSA, and KLH were used to develop precipitates. Radioautographs were prepared on Kodak RPS negative film and exposed for 2 months.

RESULTS

Early Secondary Anti-TNP Response In Vitro.—Spleen cell suspensions were placed in culture with 0.2 or 0.02 μg protein TNP-KLH (particulate or soluble) 6–7 wk following the last in vivo injection of T-K-B. (See Fig. 1 for general method). The number of cells synthesizing anti-TNP at the start of the culture and during the period of maximum response (days 3–6) is shown in Fig. 2. The number of cells producing 7S antibody is compared with the number producing 19S antibody. The higher antigen dose suppressed the 7S response 60%, whereas 19S stimulation occurred equally from both doses. Primary responses of this magnitude have not occurred under similar circumstances in our laboratory, indicating that the 19S response is a secondary one. A small

increase in cells producing anti-TNP occurred in control cultures (<100), possibly due to release of sequestered antigen (21). Experiments using un-haptenated carrier protein as antigen did not induce an anti-TNP response greater than that in control cultures. In addition, cells did not produce anti-TNP if heat-killed at 56°C for 30 min (22), if DNA synthesis was blocked by 10^{-5} M methotrexate (23), or if protein synthesis was blocked by the addition of $100\ \mu\text{g}$ chloramphenicol/culture at day 0 (24). The change in response obtained by challenge with $0.02\ \mu\text{g}$ TNP-KLH at intervals throughout the 4 month period studied is shown in Fig. 3. The general shape of the response

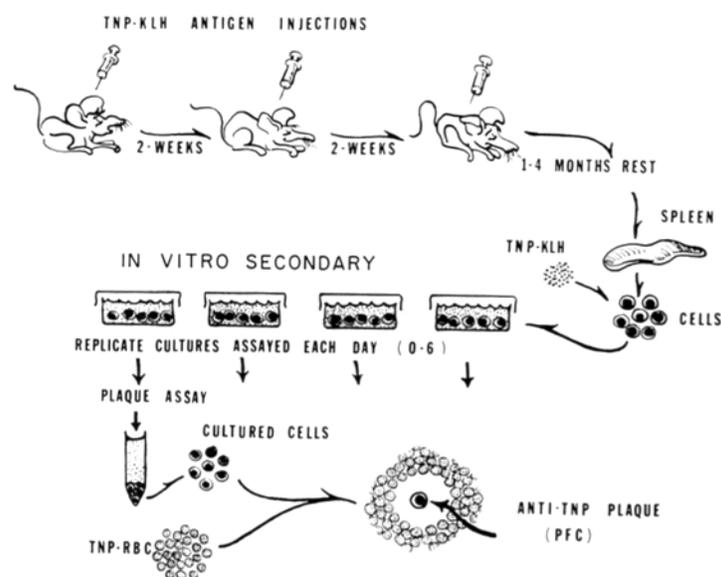


FIG. 1. Outline of culture method used to determine antigen sensitivity of memory cell population. TNP-KLH was injected or added to cultures in micrograms of protein (P) as described in the text.

curve was similar for all antigen doses tested, but peak memory varied with challenge dose.

Antibody Synthesis In Vitro.—Anti-TNP was detected in unconcentrated culture supernatants on day 6 at end point dilutions of 1:512 in antigen-stimulated cultures and 1:32 in controls using the lytic spot test. Mercaptoethanol treatment removed 19S lytic activity, but left lytic activity detectable by anti-mouse IgG unaltered. ^{14}C labeled amino acids were used to determine in vitro synthesis of antibody. Table I compares the ability of antigen-stimulated and control cultures to form plaques and to incorporate ^{14}C isoleucine and ^{14}C valine into protein and specific anti-TNP antibody. Antigen stimulation in

in vitro resulted in a 27-fold increase in anti-TNP PFC. It also caused a 16-fold increase in the incorporation of ^{14}C label into anti-TNP, as compared to non-stimulated cultures. Antigen-stimulated and control cultures incorporated approximately equal amounts of ^{14}C label into total protein. Fig. 4 shows radio-immunoelectrophoretic patterns of threefold concentrated, dialyzed super-

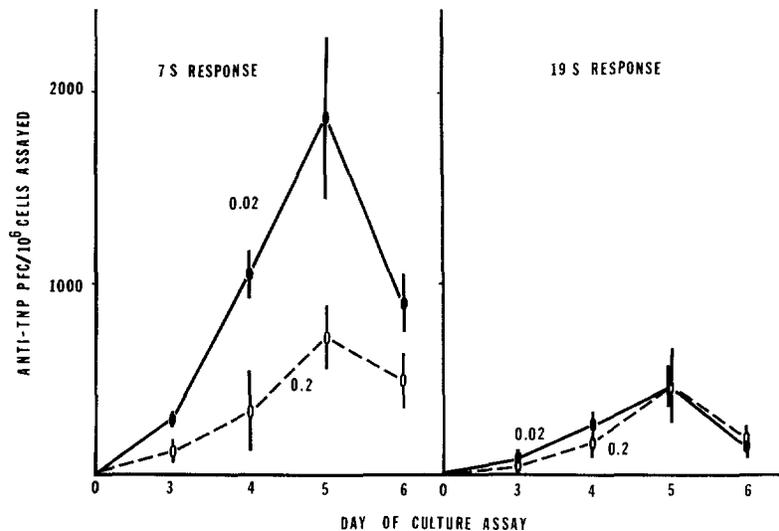


FIG. 2. Increase in cells producing anti-TNP antibody after in vitro initiation of secondary immune response. Cells were placed in culture 6–7 wk after primary immunization. Antigen was added to cultures at day 0. Either soluble or particulate TNP-KLH was used and the results were treated together, since at the same concentration both forms were shown previously to induce similar responses (11). Antigen added: 0.2 μg protein/culture $\text{O}-\text{O}$; 0.02 μg protein/culture $\bullet-\bullet$. The response was evaluated by anti-TNP plaque assay (12). Backgrounds resulting from primary stimulation have been subtracted (7S < 201 PFC/10⁶, 19S < 190 PFC/10⁶). Each point represents the mean of duplicate cultures (plated in quadruplicate) from four separate experiments; therefore, $n = 8$ except for day 4 and day 5 (0.02 μg 7S) where $n = 7$. Vertical bars represent 95% confidence intervals. 19S response was detected by direct plating. 7S response was detected by facilitation with antiglobulin serum (12).

natant from antigen-stimulated cultures. The patterns indicate ^{14}C labeled anti-TNP antibody in the supernatant. Labeled antibody was not apparent against either unhaptenated carrier. A more intense line developed with the haptenated homologous carrier protein (TNP-KLH) than with the haptenated heterologous protein (TNP-BSA), suggesting antibody against TNP-modified KLH.

7S Response: Time Dependent Development of Increased Cell Sensitivity to Antigen.—Two preliminary experiments revealed the development of in-

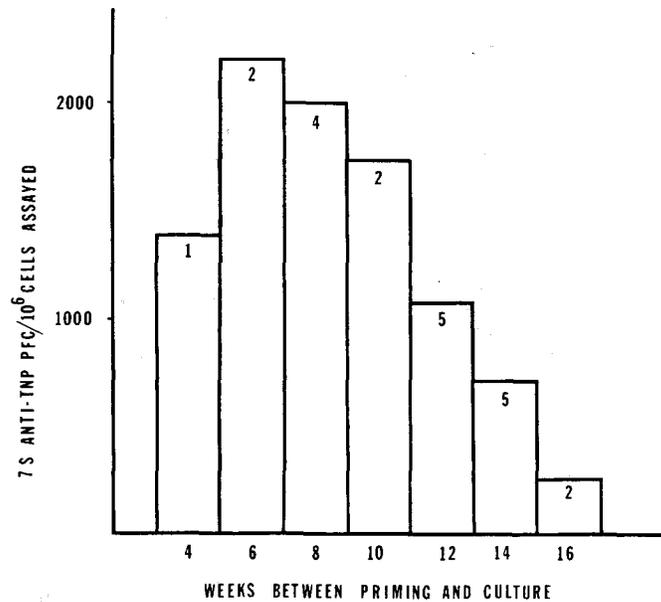


FIG. 3. Change in secondary responsiveness to a single antigen dose with time after priming. Culture and assay as described in Fig. 2. TNP-KLH (0.02 μ g protein) added on day 0 of culture. Plaque assay on day 5. Bar-enclosed numbers indicate number of individual experiments. Each experiment utilized duplicate cultures plated in quadruplicate. The height of each bar represents the mean.

creased sensitivity to antigen during the postpriming period. Six wk and 18 wk after the last *in vivo* injection of priming antigen, sets of mice were killed and their spleen cells placed in culture with log dilutions of antigen. The 7S anti-TNP PFC response obtained to each dose on day 5 is shown in Fig. 5.

TABLE I

Correlation of Secondary Anti-TNP Immune Response In Vitro. Comparison of Plaque Assay and Incorporation of ¹⁴C Isoleucine and ¹⁴C Valine into Antibody*

Culture tested	+ Antigen	- Antigen
7S PFC/10 ⁶ cells	1680	62
Net CPM in supernatant protein	3250	3428
Net CPM precipitated from supernatant [†] by TNP-BSA and Anti-BSA	491	30

* Spleen cell response to 0.002 μ g P TNP-KLH; assay on Day 5.

[†] Supernatant protein after concentration, 5.5 mg N/ml.

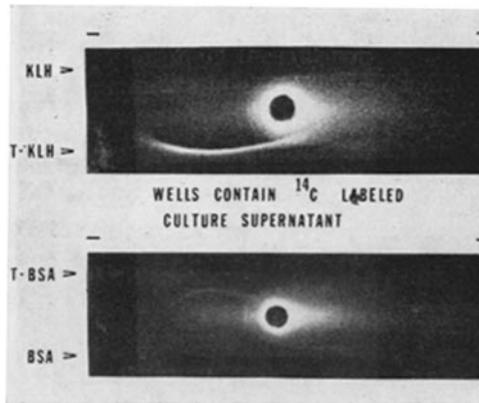


FIG. 4. Radioimmuno-electrophoretic demonstration of anti-TNP in supernatants from antigen-stimulated cultures. Supernatants were obtained and pooled on day 6 of culture and concentrated threefold prior to electrophoresis with unlabeled mouse anti-TNP-KLH antiserum as carrier. One μCi each of ^{14}C isoleucine and ^{14}C lysine was added to cultures 48 hr before assay. Cultures were stimulated by $0.002 \mu\text{g}$ TNP-KLH on day 0.

The experiments indicated: (a) The number of antibody-forming cells induced at 18 wk was one-half that at 6 wk. (b) Concentrations of antigen greater than those yielding maximum stimulation suppressed the response. The 18 wk culture was suppressed by lower antigen concentrations than the 6 wk culture. (c) Cells cultured soon after *in vivo* priming responded to a narrower range of antigen dose than cells cultured 12 wk later. (d) Cell sensitivity to antigen in-

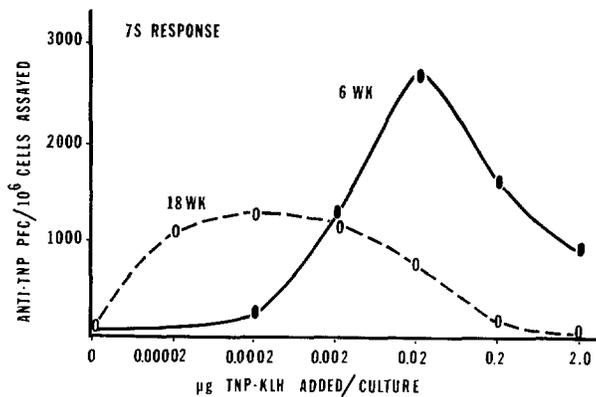


FIG. 5. Shift in anti-TNP memory cell sensitivity to antigen dose with time. Culture conditions and assay as in Fig. 2. Values are means of 7S anti-TNP PFC from duplicate cultures assayed in quadruplicate on day 5 of culture. Cells placed in culture 6 wk after priming (\bullet — \bullet); 18 wk after priming (\circ — \circ).

creased with time in vivo with maximum stimulation of 18-wk cells possible with 1000-fold less antigen than that which maximally stimulated the 6 wk group. To further investigate this latter point a series of experiments were conducted during the 6-18 wk period after priming. These experiments are summarized in Fig. 6 which reveals the continual development of increased memory-cell sensitivity during the 6-18 wk period after priming. The ratio of response (low TNP-KLH dose/high TNP-KLH dose) is given for each of eight sequential experiments. The change in ratio with time is due to increasing effectiveness of the lower antigen dose. Cell cultures from 6 wk postpriming mice were twice as responsive to the high antigen dose; however, by 10 wk both

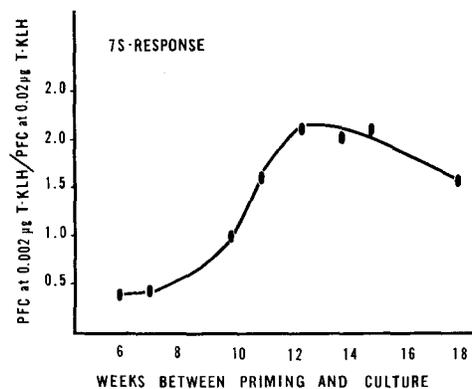


Fig. 6. Temporal transition in memory-cell sensitivity to high and low antigen doses. Relative shift in maximum anti-TNP response. Ratio of 7S response to 0.002 μg TNP-KLH/0.02 μg TNP-KLH. Each point is the mean of two cultures assayed on day 5 and plated in quadruplicate. Culture conditions and assay as in Fig. 2.

concentrations were equally effective and by 12 wk the response was completely reversed with the low dose twice as effective as the high dose. This transition in antigen sensitivity at these doses ceased at 12 wk. This cessation appeared to result from further increases in cell sensitivity concomitant with suppression by higher, previously stimulatory doses. A similar transition was obtained with 10-fold higher antigen concentrations (Table II).

19S Response: Increasing Antigen Sensitivity.—A more limited change in antigen sensitivity was observed with cells synthesizing 19S anti-TNP. 19S responses at two periods after priming are given in Table II. Cells tested early after priming were twice as responsive to the higher antigen dose. It is of interest that the 19S response of cells to low antigen concentration remained constant between the observed periods whereas the 7S response to the low antigen dose during the same interval decreased, indicating the suppression of

7S cells through increased sensitivity to antigen. In the early period IgM memory cells responded better to 10 times the antigen concentration which stimulated maximum IgG responses (Table II).

DISCUSSION

It is clear from the results that the antigen dose giving maximum recall depends on the interval between in vivo priming and in vitro challenge. Although Iványi and Černý indicated recently (25) that antigen dose is a key factor in the immune response, few systematic studies relate challenge-dose effectiveness with time after priming. It has been established that there is a critical time after priming in which a given challenge dose will produce a

TABLE II

In Vitro Anti-TNP Response to 0.2 μ g P TNP-KLH and 0.02 μ g P TNP-KLH During two Intervals after Priming*

Period after priming		21-52 Days		70-93 Days	
Antigen concentration		0.2 μ g P	0.02 μ g P	0.2 μ g P	0.02 μ g P
Mean \ddagger PFC/10 ⁶	7S	1106 \pm 346	2029 \pm 396	433 \pm 209	972 \pm 209
spleen cells	19S	695 \pm 190	548 \pm 150	354 \pm 134	518 \pm 139
Ratio:					
		$\frac{\text{PFC } 0.02 \mu\text{g P}}{\text{PFC } 0.2 \mu\text{g P}}$		$\frac{\text{PFC } 0.02 \mu\text{g P}}{\text{PFC } 0.2 \mu\text{g P}}$	
	7S	1.83		2.24	
	19S	0.79		1.5	

* Culture conditions and assay as in Fig. 2. Cells assayed on day 5 of culture.

\ddagger Mean of n cultures plated in quadruplicate \pm 95% confidence interval, where n = 16 for 0.2 μ g P, n = 15 for 0.02 μ g P (days 21-52); n = 11 for 0.2 μ g P, n = 10 for 0.02 μ g P (days 70-93). Maximum 19S background from primary stimulation was 102 PFC/10⁶ in the 21-52 day period and 160 PFC/10⁶ in the 70-93 day period. There was no detectable 7S background.

maximum anamnestic response (26) and that less antigen is required for secondary than for primary stimulation (27, 28). These in vivo and in vitro observations suggested changes in the immune memory cell population but did not reveal whether these changes were quantitative or qualitative. Our data illustrate both aspects. The memory cell population increases to a maximum and then declines in numbers but at the same time continues to change qualitatively as reflected by increasing sensitivity to lower doses. Paul, Siskind, and Benacerraf were unable to detect a consistent change in avidity for antigen leading to thymidine incorporation in cells harvested for culture either 7-19 days or 5-7 months after immunization (5). In contrast Valantová, Černý, and Iványi reported that the dose of human serum albumin required to give a maximum IgG serum titer at 16 wk after priming was 1/10 that required at 6 wk (29). However, similar increased sensitivity was not observed with SRBC

antigens after longer rest periods (30). Our results concur with and expand the findings of Valantová et al. as can be seen in Figs. 5 and 6 and in Table II. Those animals which were rested longer developed memory cells requiring less antigen in culture both for detectable threshold stimulation and for maximum stimulation.

Our ability to detect these changes may result from limiting the assay to anti-TNP PFC and/or the *in vitro* conditions. This *in vitro* model eliminates such *in vivo* complications as cell migration, antigen dilution, and catabolism or localization of antigen outside of immune tissue; however, it exposes the same tissue to antigen-antibody complexes which could be eliminated *in vivo* (31). Washing cultures at 24 or 72 hr after initiation to remove these complexes did not alter the results (32). Our data probably reflect *in vivo* processes since the 7S and 19S responses obtained *in vitro* are similar in size and kinetics to *in vivo* anti-TNP responses (12). The large IgG response obtained is typical of secondary responses. Earlier studies questioned the development of IgM memory (33, 34); however, subsequent reports have confirmed its existence (35-37). Our data confirm that IgM memory is reflected by an increase in the number of cells synthesizing antibody (37).

Passive release rather than synthesis has been reported to cause false plaques (38). Heat-killed cells in our experiments did not produce plaques, although the temperature used (56°C for 30 min) does not affect antibody; this indicates that the plaques did not result from release of passively adsorbed antibody. Furthermore, labeled anti-TNP recovered with TNP-BSA-anti-BSA precipitates and also detected with radioimmuno-electrophoresis demonstrates that antibody was synthesized *in vitro*.

Dose-response curves such as those shown in Fig. 4 are similar to those obtained by Mäkela and Mitchison *in vivo* when studying the suppression of anti-albumin memory cells in irradiated hosts (39). Failure of some investigators to detect suppressive effects with high antigen doses may have been due to overlapping optimum concentrations of the determinants on complex immunogens used to follow the immune response (40). The suppression of *in vitro* anti-TNP responses by high antigen doses, as we have noted previously (32), resembles high zone immunological tolerance described by others for a variety of antigens *in vivo* and for flagellin and endotoxin *in vitro* (41-43). Further data concerning *in vitro* tolerance to TNP are being reported separately.²

The decline in the size of the memory cell population with time depicted in Figs. 3 and 5 has been observed *in vivo* by Friedman (44) who proposed either loss of memory cells or temporary inhibition of memory resulting from antibody "feedback." Actual decline in memory cell numbers is more likely since,

² Rittenberg, M. B., and W. W. Bullock. *In vitro* initiated secondary anti-hapten response. III. Specific anti-hapten tolerance induced *in vitro*. In preparation.

in the in vitro experiments described here, cells were washed prior to culture and antibody titers in both stimulated and control cultures were relatively low. Loss of memory may have resulted from prior irreversible suppression by antibody in vivo.

With time there were fewer anti-TNP memory cells but memory cells with increased sensitivity for antigen. This leads us to conclude that only that portion of the memory cell population with high sensitivity for antigen continued to develop under in vivo antigen selection. Although we did not assay for in vivo KLH, it has been detected in rabbits 1 yr after injection (31). Furthermore, persistent stimulation by KLH was required for mouse anti-KLH memory cell development to continue for 30 days after priming (45). Consequently we interpret the results of challenge to reflect continual presence of and selective pressure by persisting immunogen. This conclusion is of theoretical interest because of the role of receptor affinity in the cell selection model proposed in recent years (1, 10, 46). This model was based on observations that with time after hapten immunization, the serum contained antibody of higher affinity. The model consists of the following proposals: (a) that cells are stimulated by contact with antigen above a threshold concentration which is related directly to the affinity of the resultant antibody (1); (b) that each cell and/or its progeny make antibody of only one affinity (1, 47); and (c) that competition for diminishing concentrations of antigen results in the selection of cells synthesizing high affinity antibody (1) and/or nonsynthesizing helper cells implicated by cell interaction studies (48-52). It is possible that such helper cells possess similar receptor sites and also undergo antigen-selected proliferation.

Most experiments supporting the antigen selection model have utilized changes detected in the affinity of the antibody produced rather than on direct demonstration of the development of cells with increased sensitivity to antigen. The temporal increase in in vitro inductive capacity of lower TNP-KLH doses reported here provides direct cellular evidence for development of cells with increased avidity for antigen, most likely reflecting the selective pressure of persisting antigen in vivo.

SUMMARY

Decreasing antigen in vivo may preferentially stimulate cells with the potential for synthesis of high-affinity antibody through activation of surface receptors with similarly high affinity. This selection should result in cells with increased sensitivity to lower antigen concentrations, cells with greater avidity for antigen. We have followed the in vivo changes in anti-TNP memory-cell sensitivity by initiating the secondary anti-hapten response in vitro. This response was determined by anti-TNP plaque assay. The results indicate that cell populations with increased sensitivity for antigen continue to emerge with time after priming and that this sensitivity may increase 1000-fold in a 4

month period. Increased sensitivity to stimulation by antigen is concomitant with suppression by higher, previously stimulatory doses as in high zone immune tolerance. The data support the hypothesis that memory cells of high avidity result from the selective pressure of diminishing *in vivo* antigen concentration.

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