Brief Definitive Report

REQUIREMENT FOR PERSISTENT EXTRACELLULAR ANTIGEN IN CULTURES OF ANTIGEN-BINDING B LYMPHOCYTES*

BY BEVERLY L. PIKE AND G. J. V. NOSSAL

(From The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, 3050 Australia)

Recent experiments from our laboratory (1-5) have described methods for the preparation of hapten-specific mouse spleen lymphocytes enriched about 500-fold in their capacity to form anti-hapten antibody in vitro. Availability of such cell populations allows a more detailed examination of interactions between immunogenic antigen and responsive cell (5). In the present study, we ask whether attachment of immunogenic antigen at the initiation of culture is, in itself, sufficient to lead to antibody formation, or whether the continuous presence of extracellular antigen is required. Previous experience with unfractionated cells would have supported the former postulate (6, 7).

Materials and Methods

Mice and Cell Suspensions. Spleen cell suspensions were prepared as previously described (3, 4) from inbred male specific pathogen-free CBA/CaH WEHI mice aged 8-10 wk and thymus cells from mice of the same strain aged 5-7 wk.

Fractionation of Hapten-Specific Spleen Cells. The hapten-gelatin method of Haas (1, 2) with the modifications described by Nossal and Pike (4) was used.

Exposure to Antigen. The "T-independent" antigen hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid–polymerized flagellin (NIP-POL) was prepared as previously described (3, 8). For pretreatment, cells were held with NIP-POL at 0°C for 30 min and washed three times through fetal calf serum. Unfractionated cells were pretreated at a concentration of 10-20 $\times$ 10^6/ml and fractionated cells at 2-3 $\times$ 10^4/ml. To minimize antigen carry-over, the tubes were changed during the washing procedure. Where antigen was continuously present, it was added at various concentrations at the initiation of culture.

Cultures. Microcultures were set up as previously described and assayed for PFC at 3 days (3, 4). Statistical analysis for PFC precursor frequency was as previously described (9). Unfractionated spleen cells were cultured at 10^6 per well either in the presence or absence of antigen, and fractionated spleen cells were cultured at or near limit dilution with 2 $\times$ 10^6 normal thymus filler cells added per well to promote the growth of single clones of antibody-forming cells. Fractionated spleen cells were cultured at 50 cells/well in the continuous presence of antigen, and pretreated fractionated cells were cultured at 200 per well in the absence of further antigen.

Results

Unfractionated spleen cells were treated with antigen for 30 min at 0°C, extensively washed, and cultured at 10^6 viable spleen cells/well in the absence of

* This is publication number 2206 from the Walter and Eliza Hall Institute of Medical Research. This work was supported by the National Health and Medical Research Council, Canberra, Australia, the National Institutes of Health grant A1-O-3958, and was in part pursuant to contract number NIH-NCI-G-7-3889 with the National Cancer Institute.

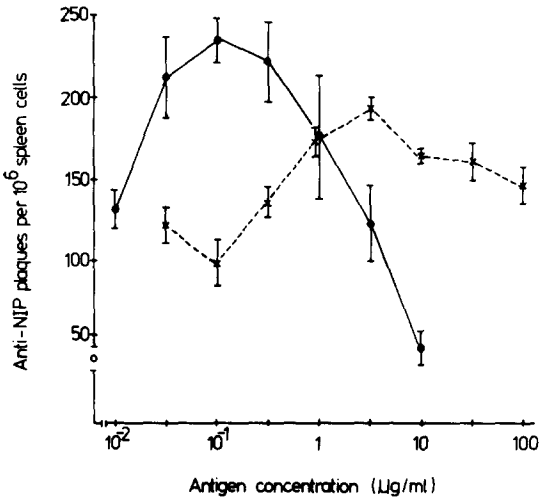

FIG. 1. Anti-NIP responses of 10^6 unfractionated CBA spleen cells after 3 days of culture either in the presence of increasing concentrations of NIP-POL (●—●) or after a single pulse of NIP-POL for 30 min at 0°C before culture (×- - -×).

further antigen. The anti-NIP responses were compared with those of cells held continuously with antigen over the full 3 days of culture (Fig. 1). As previously reported (3), cultures held continuously with antigen gave an optimal response with 30–300 ng/ml of NIP-POL and a decreased response with higher concentrations. Cultures of spleen cells which were only briefly treated with antigen gave comparable anti-NIP responses, a wide range of antigen concentrations being almost equally effective. No significant decrease in response was noted with excess antigen.

Enriched cells, cultured at limit dilution in the continuous presence of antigen, performed nearly 1,000-fold better than unfractionated spleen cells (Fig. 2). The dose-response profile was shifted somewhat to the right, but was essentially similar to that of unfractionated cells. A major difference, however, emerged with enriched cells which were pulsed only briefly with antigen, as these responded quite poorly at all antigen concentrations tested. Similarly, only poor responses (data not shown) were obtained when enriched cells were held with antigen at 37°C for periods varying from 30 min to 9 h.

In further experiments, enriched cells were pretreated with various concentrations of antigen, washed, and placed into culture with thymus cells with 100 ng/ml of NIP-POL in order to see if pretreatment of enriched cells could, by itself, cause paralysis. At no concentration between 30 ng and 100 μg/ml was any reduction below the response of control, nonpretreated cells obtained.

To investigate the possibility that pretreated fractionated cells may form small antibody-forming clones reaching their peak size before 72 h, limit dilution cultures of enriched cells, pretreated with either 300 ng/ml or 10 μg/ml of NIP-POL, were set up and assayed for anti-NIP PFC at 1, 2, and 3 days after the initiation of culture (Table I). In the complete absence of antigen at any stage, spontaneous "background" triggering of small clones occurred occasionally. Brief exposure to antigen at 0°C significantly raised this background, especially

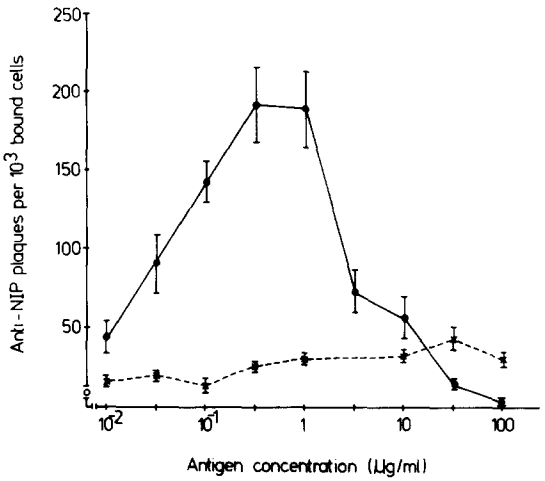

FIG. 2. Anti-NIP responses of 10^3 hapten-enriched CBA spleen cells after 3 days of culture either in the presence of increasing concentrations of NIP-POL (●—●) (50 bound cells/culture) or after a single pulse of NIP-POL for 30 min at 0°C before culture (×- - -×) (200 bound cells/culture).

TABLE I

Analysis of Rate of Growth of PFC Clones after Pretreatment with Antigen or in Its Continuous Presence

Time in culture	Antigen presentation	Frequency of precursors in bound cells*	Clonal burst size‡	Anti-NIP plaques per 10^3 bound cells
1 day	Nil	1 in 8,900	1.0	0.1
	§ Pretreatment (*a*)	1 in 750	1.9	2.6
	§ Pretreatment (*b*)	1 in 397	1.7	4.3
	‖ Continuously present	1 in 202	1.8	9.4
2 days	Nil	1 in 371	2.6	6.9
	Pretreatment (*a*)	1 in 112	2.4	21
	Pretreatment (*b*)	1 in 88	2.3	26
	Continuously present	1 in 49	3.0	62
3 days	Nil	1 in 256	3.5	14
	Pretreatment (*a*)	1 in 182	5.1	26
	Pretreatment (*b*)	1 in 172	4.8	28
	Continuously present	1 in 38	10.2	271

* For details of statistical methods see references 4 and 9.

‡ Refers to numbers of hemolytic plaques. At later days of culture, many plaques have > 1 PFC at their center, requiring a correction factor to obtain PFC numbers. See reference 4.

§ Bound cells held with 300 ng/ml (*a*) and 10 μg/ml (*b*) of NIP-POL for 30 min at 0°C, washed, and then cultured in absence of further antigen.

‖ 100 ng/ml of NIP-POL was present in the cultures throughout the culture period.

when cultures were harvested on day 1. However, even at this very early stage in clonal expansion, cultures with antigen continuously present at 100 ng/ml yielded significantly more PFC than the single brief exposure to antigen. The differences over the first 2 days of culture were due not to differences in clone size (small at these times under all circumstances) but to the proportion of enriched cells triggered into antibody formation. Over the 3rd day of culture, PFC clones exposed continuously to antigen expanded more rapidly than the few clones triggered through pre-exposure only.

Discussion

In the development of clones derived from single B lymphocytes, three elements must be considered separately. The first, which one might term activation, is highly asynchronous in both agar (10) and liquid (4) cloning systems. The second element, clonal expansion, involves sequential divisions amongst the activated cells. The third, which usually but not always (10) accompanies the second, involves differentiation of a specialized protein-secretory mechanism and initiation of large scale antibody formation. The present paper seeks to illuminate the role of antigen in these three events. We believe that previous studies using unfractionated cell populations have given misleading results. They suggest (6, 7, 11) that a simple brief encounter with antigen, initiating a single cycle of patching, capping, endocytosis, and catabolism of antigen (5), could efficiently trigger the whole cascade. However, in such studies, the carryover of antigen nonspecifically trapped in a bulky, wet cell pellet is impossible to eliminate.

The present experiments suggest that orderly clonal expansion and differentiation requires the proliferating cells to have continuous access to antigen, either free in the extracellular fluid or perhaps attached to the surface of a macrophage. Many cells placed into culture appear to be incapable of responding immediately to mitogenic signals, as evidenced by the asynchrony of clone initiation (Table I and references 4, 10, 12). Their lack of inducibility is not paralleled by a lack of appropriate antigen clearance mechanisms (5). In the case of the cells that are pulsed only briefly with antigen, it is therefore likely that antigen clearance (via capping, patching, and endocytosis [5]) will precede and thus prevent the changes leading to induction.

A further interesting finding is that transient contact with high antigen concentrations cannot cause tolerance. This was true for both unfractionated and enriched cells. Evidently functional inactivation also requires more than one cycle of signals.

Summary

A system was established to assess the requirement for continuous presence of antigen in B-lymphocyte activation to antibody formation. Mouse spleen B lymphocytes, enriched for cells bearing anti-NIP (hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid) receptors, were pretreated briefly with NIP-POL (polymerized flagellin) antigen, washed, and added in small numbers to microcultures. The behaviour of these cells was compared with that of cells cultured in the continuous presence of antigen. Unfractionated spleen cells were studied under similar conditions.

In contrast to unfractionated cells, enriched cells could not be triggered effectively by brief contact with antigen at any concentration tested. Fewer cells were activated, and clone size was smaller after brief contact with antigen than when antigen was present continuously. Furthermore, brief contact at high concentration did not cause tolerance induction.

The excellent technical assistance of Ms. Kathy Cruise and Lucy Strzelecki is gratefully acknowledged.

Received for publication 17 May 1976.

References

1. Haas, W., and J. E. Layton. 1975. Separation of antigen-specific lymphocytes. I. Enrichment of antigen-binding cells. J. Exp. Med. **141**:1004.
2. Haas, W. 1975. Separation of antigen-specific lymphocytes. II. Enrichment of hapten-specific antibody-forming cell precursors. *J. Exp. Med.* **141**:1015.
3. Pike, B. L. 1975. A microculture method for the generation of primary immune responses *in vitro*. *J. Immunol. Meth.* **9**:85.
4. Nossal, G. J. V., and B. L. Pike. 1976. Single cell studies on the antibody-forming potential of fractionated, hapten-specific B lymphocytes. *Immunology*. **30**:189.
5. Nossal, G. J. V., and J. E. Layton. 1976. Antigen-induced aggregation and modulation of receptors on hapten-specific B lymphocytes. *J. Exp. Med.* **143**:511.
6. Diener, E., and W. D. Armstrong. 1969. Immunological tolerance in vitro: kinetic studies at the cellular level. *J. Exp. Med.* **129**:591.
7. Schrader, J. W. 1974. The mechanism of bone marrow-derived B lymphocyte activation. I. Early events in antigen-induced triggering in the presence of polymerised flagellin. *Eur. J. Immunol.* **4**:14.
8. Brownstone, A., N. A. Mitchison, and R. Pitt-Rivers. 1966. Chemical and serological studies with an iodine containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology*. **10**:465.
9. Stocker, J. W. 1976. Estimation of hapten-specific antibody-forming cell precursors in microcultures. *Immunology*. **30**:181.
10. Metcalf, D., G. J. V. Nossal, N. L. Warner, J. W. Wilson, J. F. A. P. Miller, K. Shortman, T. E. Mandel, J. E. Layton, and G. A. Gutman. 1975. Growth of B-lymphocyte colonies in vitro. II. Nature of colony and colony-forming cells. *J. Exp. Med.* **142**:1534.
11. Bernstein, A., and A. Globerson. 1974. Short pulses of antigen induce in vitro an antibody response to haptenic determinants. *Cell. Immunol.* **10**:173.
12. Cunningham, B. 1974. Surface modulation: the cell surface – immunological and chemical approaches. *Adv. Exp. Med. Biol.* **51**:138.