

OVERLAP STIMULATION OF PRIMARY AND SECONDARY B CELLS BY CROSS-REACTING DETERMINANTS*

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(Received for publication 9 August 1973)

The humoral immune response to antigenic stimulation is usually typified by the elaboration of a heterogeneous, yet highly specific population of antibody molecules. In light of recent studies showing that a homogeneous antibody product is released by the progeny of a single antibody-forming cell precursor (B cell) (1, 2), the heterogeneity of serum antibody is probably a consequence of the composite antibody products of several stimulated clonal precursor cells whose individual potential antibody specificity is restricted (2, 3). Thus, the specificity inherent in the humoral immune response may be envisaged as a function of both the extensiveness of the individual's B-cell repertoire, and the selective stimulation of those precursor cells within the repertoire whose potential antibody product interacts best with the given antigen (2).

Several observations suggest, however, that the specificity of stimulation of precursor cells from previously immunized mice is less rigorous. An animal immunized initially with one antigenic determinant and exposed later to a different, but structurally similar, determinant responds to this second determinant by producing antibody with a higher affinity for the original antigen, a phenomenon referred to as "original antigenic sin" (4, 5). Similarly, when tolerance to one antigen is broken using a closely related immunogen, subsequent stimulation with the tolerogen results in the production of antibody that reacts better with the immunogen used to break tolerance (6). Such examples of anomalous stimulation by one immunogen of cells whose antibody product reacts better with a different immunogen may be a reflection of differences in the requirements for stimulation of primary and secondary precursor cells. Differences in the parameters of stimulation of primary and secondary precursor cells have been previously reported by this laboratory (2). The response of primary B cells to hapten-protein conjugates is inhibited by a concentration of free hapten 20–100 times lower than that required for equivalent inhibition of the response of secondary B cells (2). Furthermore, the affinity of antibodies released by the clonal

* Supported by research grant AI-08778 and training grants AI-00419 and CA-05022 from the U.S. Public Health Service.

† Recipient of a U.S. Public Health Service Career Development Award (I-K04-AI-33983) from the National Institute of Allergy and Infectious Diseases.

progeny of primary precursor cells shows an antigen dose dependence, i.e., antibody affinity increases as antigen concentration decreases. Secondary precursor cell stimulation, however, manifests little antigen dose dependence. These and other differences in the parameters of primary and secondary precursor cell stimulation have been interpreted as indicating that antigen receptors of primary precursor cells are functionally monovalent in their interaction with antigen, whereas receptors of secondary B cells are apparently multivalent (2). Stimulation of primary B cells would thus be more selective as a result of this functional monovalence and the consequent affinity dependence of receptor-antigen interactions.

Implicit in this hypothesis is the notion that the stimulation of primary precursor cells is rigorously determinant specific, i.e., there is little overlap frequency of primary precursor cell stimulation by closely related determinants. It would be predicted, therefore, that the population of primary precursor cells responding to the 2,4-dinitrophenyl (DNP) determinant is distinct and essentially exclusive from the population of primary precursor cells specific for the 2,4,6-trinitrophenyl (TNP) determinant. The multivalent nature of the secondary precursor cell receptors would lead to the prediction that a proportion of secondary precursor cells, stimulated originally by DNP or by TNP, may respond to subsequent stimulation by either determinant. These predictions are borne out by the findings reported here. The number of primary precursor cells responding to stimulation by a mixture of TNP and DNP conjugates of hemocyanin (Hy) is equal to the sum of the number of primary precursor cells responding to stimulation by either antigen alone. In contrast, the number of secondary B cells responding to the determinant mixture is less than the summation predicted for the stimulation of independent precursor cell populations.

Materials and Methods

Antigens.—The preparation of *Limulus polyphemus* Hy, DNP-Hy, and TNP-Hy were described previously (2). DNP-Hy and TNP-Hy contained 10 mol of hapten per 100,000 g of Hy.

Animals.—8–10-wk old Balb/c mice (Carworth Div., Becton, Dickinson, and Co., New York) were immunized by an intraperitoneal injection of 0.1 mg of DNP-Hy in complete Freund's adjuvant (CFA) and used 3–4 mo later as secondary spleen cell donors (2). Non-immune Balb/c mice were used as primary spleen cell donors at 8–10 wk of age. Balb/c recipients for cell transfers were carrier primed 4–8 wk before use by an intraperitoneal injection of 0.1 mg of Hy in CFA (2).

Cell Transfer.—Donor spleen cell suspensions were prepared in Dulbecco's modified Eagle's medium using a Teflon tissue homogenizer. Carrier-primed recipients received intravenously 2×10^6 nonimmune spleen cells or 5×10^5 immune (DNP-Hy) spleen cells 6 h after 1,000 R total body irradiation from a cesium source.

Spleen Fragment Cultures.—Primary and secondary precursor cells were enumerated by a splenic focus technique described previously (2). Spleen fragments were individually stimulated in culture with 10^{-6} M hapten (DNP or TNP) on Hy. Antihapten antibody-producing fragments were detected by a radioimmunoassay of culture fluids collected 10 and 12 days after fragment stimulation (2).

Radioimmunoassay.—The radioimmunoassay of culture fluids for anti-DNP antibody and anti-TNP antibody, and the preparation of iodinated, purified rabbit antimouse Fab fragment antibody have been described previously (2, 7). 50 μ l of culture fluid was added to 30 μ g of DNP-bromoacetyl cellulose (DNP-BAC) or TNP-human serum albumin-bromoacetyl cellulose (TNP-BAC) (7). Bound mouse antihapten antibody was detected by the binding of 125 I-labeled, purified rabbit antimouse Fab fragment antibody (2).

RESULTS

An analysis of the number of primary precursor cells specific for the DNP and TNP determinants is presented in Table I. The average number of anti-DNP antibody-producing primary foci (3.1 foci per 10^6 injected cells) and of anti-TNP antibody-producing primary foci (2.8 foci per 10^6 injected cells) are

TABLE I

The Frequency of Foci Derived from Spleen Cells of Nonimmune Mice after In Vitro Stimulation with DNP-Hy, TNP-Hy, or both DNP-Hy and TNP-Hy

Exp.	Total no. of transferred cells	Stimulating antigen*		No. of foci detected with		Total† detected foci	Frequency
		DNP-Hy	TNP-Hy	DNP-BAC	TNP-BAC		
A	8×10^6	+	—	26	21	26	$3.2/10^6$ cells
B	8×10^6	+	—	24	19	24	$3/10^6$ cells
A	8×10^6	—	+	19	23	23	$2.9/10^6$ cells
B	8×10^6	—	+	19	21	21	$2.6/10^6$ cells
A	8×10^6	+	+	43	44	49	$6.1/10^6$ cells
B	8×10^6	+	+	41	45	48	$6/10^6$ cells

* All antigenic stimulation in vitro at 10^{-6} M haptenic determinant concentration.

† Total of all fragment cultures detected as positive with either DNP-BAC or TNP-BAC (fragment cultures positive for both DNP and TNP are counted once).

similar to those reported previously (2)¹ and do not change significantly after a 10-fold increase or decrease in hapten determinant concentration (2).¹ The number of primary foci obtained after fragment stimulation with a mixture of TNP-Hy and DNP-Hy (6 foci per 10^6 injected cells) approximates the sum of the numbers obtained from single determinant stimulation (5.9 foci per 10^6 injected cells). Thus, the TNP determinant appears not to stimulate the pool of primary precursor cells specific for DNP, and vice versa, although the presence of a small percentage of overlap stimulation of these pools cannot be ruled out by these data.

Table II presents the results of an analysis of the stimulation by TNP-Hy and/or DNP-Hy of precursor cells from mice previously immunized with DNP-Hy. 11.8 foci per 10^6 injected cells were detected after DNP-Hy stimulation of such secondary precursor cells (2). Stimulation with TNP-Hy of B cells ob-

¹ Press, J. L., and N. R. Klinman. 1973. Manuscript submitted for publication.

TABLE II
The Frequency of Foci Derived from Spleen Cells of Mice Immunized with DNP-Hy after In Vitro Stimulation with DNP-Hy, TNP-Hy, or both DNP-Hy and TNP-Hy

Exp.	Total no. of transferred cells	Stimulating antigen*		No. of foci detected with		Total† detected foci	Frequency
		DNP-Hy	TNP-Hy	DNP-BAC	TNP-BAC		
A	4×10^6	+	-	48	42	48	$12/10^6$ cells
B	4×10^6	+	-	46	41	46	$11.5/10^6$ cells
A	4×10^6	-	+	26	28	28	$7/10^6$ cells
B	4×10^6	-	+	24	25	25	$6.3/10^6$ cells
A	4×10^6	+	+	51	43	56	$14/10^6$ cells
B	4×10^6	+	+	47	40	52	$13/10^6$ cells

* All antigenic stimulation in vitro at 10^{-6} M haptenic determinant concentration.

† Total of all fragment cultures detected as positive with either DNP-BAC or TNP-BAC (fragment cultures positive for both DNP and TNP are counted once).

tained from DNP-Hy-immunized mice yielded 6.7 antihapten-producing foci per 10^6 injected cells. This is 2.4 times more than the number detected after TNP-Hy stimulation of precursor cells from nonimmunized mice and indicates that some precursor cells stimulated originally by DNP can be stimulated secondarily by TNP to give a focal response. This conclusion is reinforced by the finding (Table II) that the number of secondary foci detected after stimulation with a mixture of TNP-Hy and DNP-Hy (13.5 foci per 10^6 injected cells) is appreciably less than the number predicted (18.5 foci per 10^6 injected cells) if separate precursor cell populations (for TNP and DNP) were responding to secondary stimulation. The fact that the combination of DNP-Hy and TNP-Hy stimulates more precursor cells than DNP-Hy alone probably indicates that TNP-Hy also stimulates TNP specific primary precursor cells within the immune donor cell population.

DISCUSSION

Previous reports from this laboratory have indicated the single-cell origin of primary and secondary splenic foci derived under the experimental conditions utilized in this study. The finding that the number of foci detected is linearly related to the number of injected cells (1, 2) and is independent of the presence of theta antigen-bearing cells (8), and that the antibody produced by such foci appears homogeneous by the criteria of isoelectric spectra (2, 9), chain recombination (10), and hapten-binding properties (1, 2, 10) suggests that such foci are derived from the clonal progeny of a single stimulated antibody-forming cell precursor. These studies also indicated that the stimulation of primary precursor cells exhibits relatively greater antigen dose dependence than the stimulation of secondary precursor cells, and that whereas primary precursor cells have an affinity threshold for stimulation, secondary precursor cells may not (2).

The finding in this report that there is a disparity in the degree of overlap stimulation of primary vs. secondary precursor cells by closely related determinants confirms the notion that the specificity of stimulation of primary precursor cells is more rigorous than that of secondary precursor cells (2). It is unlikely that this is due to differences in antibody affinity or immunoglobulin class, since (a) the majority of anti-DNP antibody produced by either primary or secondary foci is IgG1 (11), and (b) there is less than a 10% difference in the average binding energy of primary vs. secondary monofocal anti DNP antibodies (2). The observed disparity in overlap stimulation of primary and secondary cells is consistent, however, with previously observed differences in the parameters of stimulation of these cells, and is more likely explained by the differences postulated for the functional receptor valence of primary and secondary precursor cells. The monovalent interaction of primary cell receptors with antigen results in a greater affinity dependence of stimulation; whereas, the multivalent thus highly avid nature of secondary precursor cell receptors obviates, to some extent, affinity-dependent interactions (2). Phenomena such as "original antigenic sin" can thus be correlated with ease of secondary precursor cell stimulation by closely related determinants, and are explained by the multivalent, hence relatively affinity independent, secondary cell receptor interactions with antigen. It follows that the production of anti-DNP antibody by animals given a secondary immunization with TNP-protein conjugates after primary immunization with DNP-protein conjugates is a consequence of the stimulation of DNP-specific secondary precursor cells by TNP. The extent to which such overlap stimulation of secondary B cells accounts for other instances of "original antigenic sin," such as that observed in responses to closely related viruses (12), is not yet clear.

The qualitative differences between primary and secondary B cells noted here and in other reports (2, 11, 13) strongly militate against the idea that antigen plays a role as a positive selective force in a somatic generation of B-cell diversity. Exposure to antigen would be expected to result in the production of cells that behave as secondary precursor cells, and thus primary cell behavior should not be observed. In fact, the studies reported here demonstrate an exquisitely specific primary immune response. Although the multivalent antibody produced by more than 80% of primary anti-DNP clones can bind to TNP-BAC, few of these clones can be stimulated by exposure to TNP-Hy. The affinity of interaction between primary cell receptor and immunogen is thus an important parameter of primary cell stimulation. Only interactions that exceed a minimum monovalent affinity threshold appear to be stimulatory. Studies clarifying the nature of this affinity threshold may shed critical light upon the question of specificity and overlap in the primary immune response.

SUMMARY

Experiments were carried out to test the validity of the hypothesis that postulated differences in the nature of the antigen receptors of primary and secondary

B cells should be reflected in a greater specificity in primary B-cell stimulation (2). Enumeration of clonal precursors stimulated by either DNP-Hy, TNP-Hy, or a mixture of both antigens confirmed this hypothesis. Since the sum of primary B cells stimulated by DNP-Hy and TNP-Hy is approximately equal to the number stimulated by a mixture of both, overlap stimulation of primary B cells by these antigens could be considered negligible. In contrast, the stimulation of B cells from mice previously immunized with DNP-Hy showed extensive overlap of stimulation by DNP-Hy and TNP-Hy. Thus secondary B cells appear less fastidious in their affinity requirements for stimulation than primary B cells.

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