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Fetal Lymphoid Tissues: Increase in Antigen-Binding Cells Induced *in Vitro* by Antigen, LPS, and Aggregated IgG[1] (39395)

MARVIN L. TYAN

Dental Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Previous work has shown that by the tenth day of gestation, embryonic tissues involved in the ontogenesis of the mouse immune system (1, 2) contain small numbers of antigen-binding cells (ABC), specific for several antigenic determinants (3). Cells from these tissues, like those from adult lymphoid tissues, respond to antigen *in vitro* with proliferation (4), an increase in the number of cells bearing IgM, IgG, and IgA on their membranes (4, 5), and with the production of small quantities of specific antibody (4, 6).

Presented here are observations showing that when mouse fetal liver cells were cultured with antigen, specific ABC were formed and the number produced was increased if lipopolysaccharide (LPS) or aggregated human IgG had been added to the cultures; despite this increase in ABC there was no detectable increase in the number of antibody-secreting cells (plaque-forming cells, PFC). By contrast, LPS and aggregated IgG had no significant effect on the numbers of ABC in antigen-stimulated cultures of adult spleen cells, but they did have profound effects on the numbers of PFC generated in these cultures. These findings suggest that the appearance of specific antibody on the membranes of B cells or their precursors and the ability to secrete significant quantities of this antibody represent separate steps in the maturation of B cells.

Materials and methods. Tissue donors. Fetal liver and adult spleen and thymus cells were obtained from CBA/J mice (Jackson Laboratories, Bar Harbor, Maine). Embryonic age was determined by considering the day the vaginal plug was observed as day zero.

Culture conditions. Cells from adult mice were dissociated, washed, and added to the cultures described below at a concentration of 5×10^6/ml (4). Fetal liver cells from a single pregnancy were pooled; the cells were dissociated and added to cultures at concentrations of 1-to-10 $\times$ 10^6/ml. The medium was Eagle's S-MEM, with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin (100 units and 100 μg/ml, respectively).

Primary responses to sheep red cells (SRBC) were elicited as described previously (4). Briefly, an underlay of washed SRBC (2%) in 1.5 ml of agar (0.25%) was placed in 35 $\times$ 10-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and 4 ml of cell suspension was added after the agar had hardened. Where indicated, heat-aggregated human IgG (63° for 20 min, 200 μg/ml; Miles Laboratories, Kankakee, Ill.) or LPS *Escherichia coli* B5 (5 μg/ml; Difco, Detroit Mich.) was added, and the cells were cultured for 3 days at 37° in 5% CO_2 in air. At this point the cells were removed from the plates, washed three times in a Ca- and Mg-free, buffered salt solution (PBS), and resuspended at a concentration of 25 $\times$ 10^6/ml. The SRBC-agar underlay were processed for areas of complement-dependent hemolysis (hemolytic foci; (4)). All cultures were done in triplicate. The aggregated human IgG did not produce agglutination of SRBC when tested over a wide range of concentrations.

SRBC-rosettes (ABC). The detection of cells specifically binding SRBC (ABC) was performed by standard methods (7, 8; cells having Fc and complement receptors were also determined but the results are not reported here). Briefly, 0.2 ml of cell suspension was added to 0.5 ml of PBS, and 0.1 ml of washed SRBC (1%). The cells were gently centrifuged, carefully resuspended, and examined in a hemocytometer under 400$\times$ magnification. A minimum of 2000

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nucleated cells were scanned. The specificity of ABC formation was demonstrated by culturing cells with and without SRBC and testing with SRBC and horse RBC, and vice versa. Unlike human T cells, mouse cells do not form nonspecific rosettes with SRBC.

Because the addition of LPS or aggregated IgG to cultures often produced an increase or decrease in proliferation, in each experiment the frequencies of ABC in cultures containing these agents were corrected as follows: observed frequency × total cells recovered: total cells recovered from cultures containing only SRBC. The correction factors (mean and range) were (a) adult spleen: LPS, 1.3 (0.75–2.0); IgG, 0.9 (0.75–1.1), (b) fetal liver: LPS, 0.96 (0.7–1.2); IgG, 1.3 (0.9–2.2). The results from 19 experiments were pooled and analyzed by means of Student's *t* test.

PFC. In six experiments the cells recovered from the cultures were assayed for direct PFC by a slide modification of the Jerne technique (4).

Immunofluorescent studies. Cells obtained from cultures and SRBC-rosettes were examined for membrane-bound IgM by the indirect immunofluorescent method on fixed preparations. The anti-IgM serum reacted specifically with the heavy chain of mouse IgM, and neither antisera cross-reacted with fetal calf serum or human IgG (Meloy Laboratories, Springfield, Va.). Mouse thymocytes did not bind either antiserum.

Results. When tested before culture very few cells from adult spleen-bound SRBC (0.1 to 0.4%, data not shown). After 3 days in culture with SRBC, the frequency of ABC either did not change or, at most, increased threefold (Table I). The addition of LPS or aggregated IgG to the cultures did not consistently or significantly alter the frequency of SRBC-ABC; on the other hand, the numbers of hemolytic foci and PFC produced in these cultures were increased by LPS and decreased by IgG to significant degrees (Table II). Membrane-bound IgM was easily detected by immunofluorescent studies on SRBC-ABC taken from cultures of adult spleen, and the intensity of staining was only modestly, if at all, increased on cells taken from cultures containing LPS or IgG.

By contrast, no SRBC-ABC were detected with certainty among fetal liver cells before culture (i.e., <1:10,000); but after 3 days *in vitro* the numbers of specific SRBC-ABC significantly exceeded those recovered from cultures of adult spleen (Table I). The addition of LPS or aggregated IgG to these cultures resulted in further increases in the formation of ABC. More cells were recovered from cultures containing IgG than from the controls (up to 220%), but LPS had no significant effect on the number of cells recovered. Liver cells cultured with SRBC formed rosettes with SRBC but not with horse RBC, and vice versa.

As was the case with adult spleen cells, IgM was readily detected on the membranes of SRBC-ABC taken from cultures of fetal liver, but, in contrast, the presence of LPS or IgG in the medium resulted in a dramatic increase in the intensity of staining. However, neither LPS nor IgG significantly altered the number of PFC recovered from cultures of fetal tissues (Table II). As previ-

TABLE I. FREQUENCY OF SPECIFIC ANTIGEN-BINDING CELLS AMONG FETAL LIVER, ADULT SPLEEN AND ADULT THYMUS CULTURED FOR 3 DAYS WITH SRBC IN THE PRESENCE OR ABSENCE OF AGGREGATED HUMAN IgG (200 μg/ml) OR LPS (5 μg/ml)

Agent added to culture	Percentage of cells binding SRBC (± SD)[a]							
	Age of gestation						Adult spleen	Adult thymus
	9–10	11–12	13–14	15–16	17–18	19–20		
None	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.3	1.7 ± 0.5	1.9 ± 0.6	0.9 ± 0.4	0.7 ± 0.6[c]	<0.001
Aggregated IgG		0.5 ± 0.1	2.1 ± 0.8[b]	3.4 ± 0.6[b]	3.6 ± 1.1[b]	1.7 ± 0.9	0.8 ± 0.7[c]	<0.001
LPS			1.6 ± 0.2	2.7 ± 0.4[b]	2.2 ± 0.5	1.5 ± 0.3	0.5 ± 0.6[c]	<0.001

[a] These data have been compiled from 19 separate experiments, and in each experiment the frequencies of ABC in cultures containing aggregated IgG or LPS have been corrected as follows: observed frequency × total cells recovered: total cells recovered from cultures containing only SRBC.

[b] $P < 0.05$ (Student's *t* test), when compared to the number of ABC among cells cultured with SRBC only.

[c] $P < 0.01$, when compared to values obtained with fetal liver cells from 13- to 20-day embryos cultured under the same conditions.

TABLE II. DEVELOPMENT OF HEMOLYTIC FOCI AND PFC BY ADULT SPLEEN AND FETAL LIVER CELLS CULTURED WITH SRBC IN THE PRESENCE OR ABSENCE OF AGGREGATED HUMAN IgG (200 μg/ml) OR LPS (5 μg/ml)[a]

Source of cells	SRBC in culture	Agent added	Number per 10^6 cells (± SD)	
			Hemolytic foci	PFC
Adult spleen	No		0	0
	Yes		12.2 ± 1.2	117 ± 10.4
	Yes	Agg.IgG	3.8 ± 0.9	19 ± 3.5
	Yes	LPS	16.7 ± 2.3	245 ± 30.6
Fetal liver (14 to 18 day)[b]	No		0	0
	Yes		0[c]	16.2 ± 5.2
	Yes	Agg.IgG	0[c]	12.1 ± 6.1
	Yes	LPS	0[c]	19.7 ± 3.4

[a] These data have been compiled from six experiments.
[b] Age of gestation.
[c] The agar in these plates contained from 4 to 10 defects approximately 1 mm in diameter that could represent complement-dependent hemolysis and/or enzymatic damage from overlying cells.

ously reported (4) the hemolytic plaques formed by fetal cells were small and few, but they were demonstrated to be specific for SRBC (i.e., no response against horse RBC). In addition, the plaques were complement dependent, and they could be completely inhibited by adding anti-IgM serum (1:40) or sodium azide (0.1%) to the slides. No hemolytic foci were identified with certainty on the agar underlay.

Discussion. Under the experimental conditions described here, when cells from adult mouse spleen were cultured with SRBC the frequency of specific ABC increased little, if at all; the addition of LPS or aggregated IgG to these cultures did not alter this. On the other hand, LPS increased and IgG decreased significantly the numbers of antibody-secreting cells recovered from these cultures. By contrast, under the same conditions, fetal lymphoid precursors produced relatively large numbers of ABC and this was further increased by the addition of LPS or IgG. Nevertheless, despite evidence from immunofluorescent studies of increased IgM on the membranes of ABC taken from cultures containing LPS or IgG (9, 10), these agents did not alter the number of antibody-secreting cells demonstrable in cultures of fetal tissue. Taken together, these findings suggest that the appearance of specific antibody on the membranes of B cells or their precursors and the ability to secrete significant quantities of this antibody represent separate steps in the maturation of B cells (1).

The increase in ABC induced by aggregated IgG and LPS in cultures of antigen-stimulated fetal liver cells was unexpected, and the mechanisms of action remain unclear. In these and in previous experiments (12) the addition of aggregated IgG to cultures of adult spleen cells resulted in no significant change in the frequency of ABC, but in up to 95% inhibition of PFC and of the binding of antigen-antibody complexes. While this suggests that the Fc receptor on B cells may be involved in the inhibition of PFC by IgG (13), it is not clear that the same mechanism is involved in modulating the frequency of ABC. The mode of action of LPS on fetal cells is even more obscure; it does not appear to be mitogenic for B cell precursors (14), and this suggests the possibility that its effect on ABC may be indirect.

Summary. When adult spleen cells were cultured with SRBC, the frequency of specific antigen-binding cells (ABC) either did not change or, at most, increased threefold. The addition of LPS or aggregated IgG to the cultures did not alter this, although the numbers of antibody-secreting cells recovered from the cultures were markedly increased by LPS and decreased by IgG. In contrast, when fetal liver cells were cultured with SRBC and LPS or aggregated IgG, the numbers of SRBC-ABC formed exceeded that found in cultures of adult cells, but there was no change in the small number of PFC produced.

1. Tyan, M. L., J. Immunol. **100,** 535 (1967).

2. Tyan, M. L., and Herzenberg, L. A., J. Immunol. **101,** 446 (1968).
3. Decker, J. M., Clarke, J., Bradley, L. M., Miller, A., and Sercarz, E. E., J. Immunol. **113,** 1823 (1974).
4. Tyan, M. L., Ness, D. B., and Gibbs, P. R., J. Immunol. **110,** 1170 (1973).
5. Owen, J. J. T., Cooper, M. D., and Raff, M. C., Nature (London) **249,** 361 (1974).
6. Tyan, M. L., Herzenberg, L. A., and Gibbs, P. R., J. Immunol. **106,** 1283 (1969).
7. Bianco, D., Patrick, R., and Nussenzweig, V., J. Exp. Med. **132,** 702 (1970).
8. Möller, G., J. Exp. Med. **139,** 969 (1974).
9. Pernis, B., Forni, L., and Amanti, L., Ann. N.Y. Acad. Sci. **190,** 420 (1971).
10. Melchers, F., and Andersson, J., Eur. J. Immunol. **4,** 191 (1974).
11. Andersson, J., Buxbaum, J., Citronbaum, R., Douglas, S., Forni, L., Melchers, F., Pernis, B., and Stott, D., J. Exp. Med. **140,** 742 (1974).
12. Tyan, M. L., Proc. Soc. Exp. Biol. Med. **150,** 313 (1975).
13. Basten, A., Miller, J. F. A., P., Sprent, J., and Pye, J., J. Exp. Med. **135,** 610 (1972).
14. Kearney, J. F., and Lawton, A. R., J. Immunol. **115,** 677 (1975).

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