Proc. Nat. Acad. Sci. USA
Vol. 69, No. 6, pp. 1440–1444, June 1972

Development of Cellular Immunity in the Human Fetus: Dichotomy of Proliferative and Cytotoxic Responses of Lymphoid Cells to Phytohemagglutinin

(T and B cells/DNA synthesis/target cells)

DANIEL P. STITES, MARTIN C. CARR, AND H. HUGH FUDENBERG

Section of Hematology and Immunology, Department of Medicine, and Department of Obstetrics and Gynecology, School of Medicine, University of California, San Francisco, Calif. 94122

Communicated by Philip Levine, March 22, 1972

ABSTRACT **The reactivity of cells *in vitro* was investigated with specimens from various lymphoid organs of seven human fetuses. Thymocytes responded to stimulation by phytohemagglutinin with significant increases in synthesis of DNA, but failed to produce destruction of xenogeneic target cells. In cells from bone marrow, precisely the converse pattern of reactivity to the mitogen was detected. Lymphocytes from spleen and peripheral blood demonstrated both phytohemagglutinin-dependent functions, while hepatic cells did not respond to phytohemagglutinin. Based on the striking dichotomy of phytohemagglutinin-dependent responses in fetal thymocytes and bone-marrow lymphoid cells, we conclude that phytohemagglutinin-dependent cytotoxicity and DNA synthesis are functions of different populations of lymphoid cells during human embryonic development.**

Activation of lymphocytes by the plant mitogen, phytohemagglutinin (PHA), results in a complex series of biochemical events that closely mimic antigenic stimulation of immunocompetent cells *in vitro* (1). Effects of PHA on susceptible lymphocytes include the *stimulation of DNA synthesis* and the *induction of nonspecific cytotoxicity for target cells* (1). The cytotoxicity is nonspecific since this PHA-induced response does not require prior sensitization of lymphocytes as does specific antigen-induced cytotoxicity; the PHA activation is a useful *in vitro* model for the study of lymphocytotoxicity (2–5).

The proliferation of lymphocytes that is induced by PHA is known to be primarily a property of thymus-derived cells (6–8), but the precise mechanism and immunologic significance of this reaction is not yet clear. Lymphoid cells capable of mediating cytotoxic reactions *in vivo* are important in surveillance against tumor cells, rejection of tissue grafts, and in autoimmunity (9).

PHA-induced synthesis of DNA and nonspecific target-cell destruction have been studied in various animal and human lymphoid cells in health and disease (1). Although limited data are available on PHA-induced synthesis of DNA (10–12), no studies on lymphocytotoxicity *in human fetal lymphoid cells* have been reported. The delineation of the cell types that mediate these PHA-dependent reactions in the developing human fetus should provide a better understanding of the thymus-dependent (T cell) and bone marrow-dependent (B cell) lymphocyte populations in adult man. This knowledge may offer insight into the apparent immunologic paradox of the mammalian pregnancy and may help in determining the ontogeny of cellular immunity in man.

In the present study, we have investigated in human fetal lymphoid cells the proliferative response to PHA and the PHA-induced xenogeneic target-cell destruction. A striking independence of these two phenomena was found, especially in cells obtained from thymus and from bone marrow. On exposure to PHA, fetal thymocytes exhibited marked increases in synthesis of DNA while producing little or no target-cell destruction, whereas fetal bone-marrow cells demonstrated precisely the opposite pattern.

Abbreviations: PHA, phytohemagglutinin; PBS, phosphate-buffered saline.

MATERIAL AND METHODS

Preparation of Suspensions of Lymphoid Cells. Human fetuses of conceptional ages between 14 and 18 weeks, as gauged by crown-rump length, were obtained by abdominal hysterotomies performed for therapeutic abortions. By this stage of development the thymus contains a well-demarcated medulla and cortex; circulating blood lymphocytes are present; the splenic white pulp is sparse, but the liver contains large numbers of lymphoid cells. The thymus, spleen, and liver were teased, minced with sterile scalpels, and gently homogenized in a glass tissue grinder within 1 hr after delivery. Bone-marrow cells were flushed from the epiphyseal ends of long bones with phosphate-buffered saline, pH 7.4 (PBS), and suspended in the same buffer. Peripheral blood collected in phenol-free heparin (Lipo-hepin, Riker Laboratories) was placed on a gradient of Ficoll–Hypaque and centrifuged at 400 × *g* for 20 min according to the method of Böyum (13); the lymphocytes contained in the flotation ring were removed with a sterile Pasteur pipette. Clumps in all cells suspensions were removed by rapid filtration through a sterile syringe packed with glass wool. All cells were washed twice in PBS by centrifugation at 400 × *g*. The cell suspensions contained more than 95% mononuclear cells, and 95–100% were viable at the initiation of experiments (by trypan blue exclusion test).

Cultures of Lymphoid Cells for DNA Synthesis. A modification of the microculture method of Hartzman *et al.* (14) was used. 2×10^5 cells were placed into 12 × 75-mm plastic tubes (Falcon no. 2054) containing 1.0 ml of medium 199 with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) (pH 7.2) penicillin (100 units/ml), streptomycin (100

μg/ml), 20 mM fresh L-glutamine (Grand Island Biological Co.), and 20% human AB serum. PHA-M (control no. 546514, Difco) was weighed, dissolved in PBS, sterilized by filtration through a 0.45-μm Millipore filter, and stored at −20° until use. Doses of PHA ranging from 3 to 1000 μg (in 0.1 ml of PBS) were added to each culture tube. Control tubes were prepared without PHA. Triplicates for each observation were incubated for 66 hr at 37° in 5% CO_2–95% air. 2 μCi of tritiated thymidine (16 Ci/mmol) (Schwarz/Mann) were added, and incubation was continued for 6 hr.

Preparation of Cells for Scintillation Counting. Cell suspensions were transferred from plastic culture tubes to 16 × 100-mm glass tubes, washed 3 times with cold PBS, and centrifuged at 500 × *g* for 20 min. DNA protein was precipitated twice with cold 5% trichloroacetic acid. Precipitates were washed with cold absolute methanol and dried. The trichloroacetic acid–methanol insoluble precipitates were dissolved in 0.2 ml of 0.2 N NaOH by heating to 60° for 45 min. 0.2 ml of 10% acetic acid was added, and the radioactive material was transferred to scintillation vials containing 10 ml of Liquifluor–toluene (Packard) with 10% Bio-Solv (BBS-3, Beckman). Counts per minute (cpm) were determined in a Packard liquid scintillation counter with an automatic external standard for quench correction. Results are expressed as the mean cpm of triplicate tubes for each dose of PHA and for each set of control tubes. The stimulation index for each dose of PHA is derived by the expression:

$$\frac{\text{mean cpm in PHA-treated cultures}}{\text{mean cpm in control cultures}}.$$

Cytotoxicity of Lymphoid Cells for Chicken Erythrocytes. Chicken erythrocytes were obtained, prepared, and labeled with $Na_2{}^{51}CrO_4$ according to the method of Perlmann *et al.* (15). Briefly, heparinized chicken erythrocytes obtained by cardiac puncture were washed in PBS, and the pellet, containing about 3 × 10^7 cells, was incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham/Searle) for 45 min at 37°. After five washes in PBS, the chicken erythrocytes were adjusted to a density of 2 × 10^5/ml in Eagle's minimal essential medium (spinner modification, Grand Island Biological Co.) containing 10% heat-inactivated fetal calf serum.

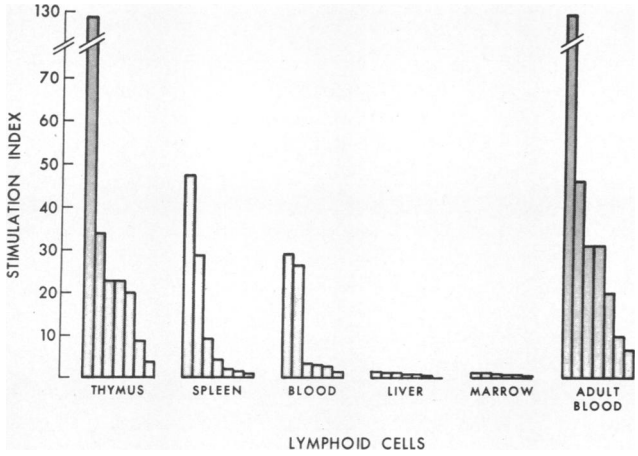

FIG. 1. Incorporation of [^{3}H]thymidine into DNA of PHA-stimulated fetal lymphoid organs. Stimulation index, mean cpm in PHA-treated cultures/mean cpm in control cultures.

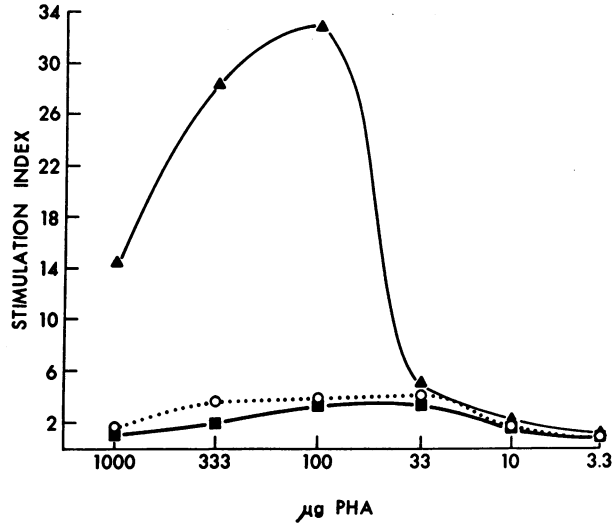

FIG. 2. Dose-response curve of DNA synthesis in various fetal lymphoid cells stimulated with PHA. This fetus was of a conceptional age of 15 weeks. ▲—▲, thymus; O···O, spleen; ■——■, blood.

The cytotoxicity assay was performed in 13 × 100-mm screw-capped plastic tubes (Falcon no. 2027). To each of three replicate tubes was added 0.5 ml of the suspension of ^{51}Cr-labeled chicken erythrocytes (10^5 cells) and 0.5 ml of a suspension of fetal lymphoid cells containing 10^6 or 5 × 10^6 cells. 800 μg of PHA in a volume of 0.1 ml were added to selected tubes. Control tubes contained chicken erythrocytes with or without PHA or chicken erythrocytes and lymphoid cells. 2 × 10^6 unlabeled human erythrocytes were added to each tube, since this has been shown to stabilize the system (15). Finally, the volume of each tube was adjusted to 1.5 ml with medium. The tightly capped tubes were incubated at 37° in air for 21 hr and centrifuged at 200 × *g* for 5 min. 1 ml of the supernatant was removed and counted in a Packard Auto-Gamma spectrometer with a well-type scintillation detector.* The results are expressed as follows:

(*a*) % ^{51}Cr release (chicken erythrocytes + lymphoid cells) =

$$\frac{\text{cpm (chicken erythrocytes + lymphoid cells)} - \text{cpm (chicken erythrocytes)}}{\text{total cpm of pellet}}$$

(*b*) % ^{51}Cr release (chicken erythrocytes + lymphoid cells + PHA) =

$$\frac{\text{cpm (chicken erythrocytes + lymphoid cells + PHA)} - \text{cpm (chicken erythrocytes + PHA)}}{\text{total cpm of pellet}}$$

RESULTS

DNA synthesis in lymphoid cells exposed to PHA

Lymphoid cells from seven fetuses were assayed for incorporation of [^{3}H]thymidine in response to various doses of PHA.

* For determination of net releasable counts, 1.5 ml of distilled water was added to a washed pellet of 10^5 chicken erythrocytes; 1 ml of the supernatant was counted. The counts released by water hemolysis generally represented 63–65% of the counts detectable in the pellet.

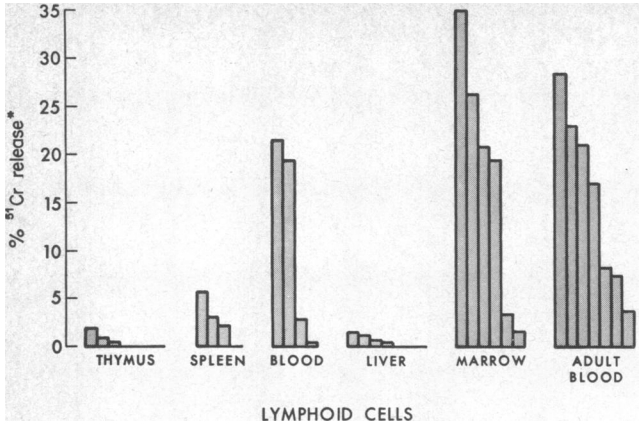

FIG. 3. PHA-mediated destruction of xenogeneic target cells (chicken erythrocytes). No (0%) release of ^{51}Cr was observed in four thymocyte, one splenocyte, and three hepatocyte cultures. * ^{51}Cr release: chicken erythrocytes + lymphoid cells + PHA.

The stimulation indices, which correspond to the maximum dose response to PHA in various lymphoid cells, are shown in Fig. 1. Thymocytes responded with maximum stimulation indices ranging from 3.9 to 128, similar to the range observed in control adult lymphocytes. Splenocytes and peripheral blood lymphocytes had a somewhat lower response, although the mean responses of cells from these two organs were not statistically different from the response of thymocytes. In contrast, hepatocytes and bone-marrow cells showed essentially no response to PHA. Mean counts per minute (± standard error) in unstimulated cultures were (Fig. 1): thymus, 431 ± 107; spleen, 892 ± 148; blood, 13400 ± 5267; marrow, 19200 ± 3020; liver, 885 ± 212. The high "background" DNA synthesis in control bone-marrow cultures may have obscured any measurable increment in DNA synthesis caused by PHA, but the comparable "high background" in control blood cultures did not.

The dose of PHA required for maximum DNA synthesis in the three responding organs (thymus, spleen, and blood) was about 100 μg of PHA per 2×10^5 cells per ml of culture. A typical dose–response curve for thymocytes, splenocytes, and blood lymphocytes from a 15-week fetus is shown in Fig. 2. There was inhibition or lack of response both at the larger and smaller doses of PHA. Due to the relatively small sample size, we were unable to establish an age-dependent effect on DNA synthesis that is induced by PHA.

PHA-mediated toxicity of lymphoid cells for chicken erythrocytes

When lymphoid cells from the same fetal tissues were assayed for PHA-stimulated cytotoxicity at a ratio of ten lymphoid cells to one target cell (Fig. 3), an entirely different pattern of responses emerged (Table 1). Significant release of isotope occurred in five of six fetal marrow-cell preparations (mean of significant releases, 21%), three of four fetal blood (14.6% released), and six of seven adult blood lymphocytes (17.5%). Three of four samples of fetal splenocytes produced statistically significant ^{51}Cr release, but at a lower level (Table 2). In contrast, significant isotopic release occurred in only one of six thymocyte and hepatocyte cultures. In each of these single instances, the release was between 1 and 2%. By increasing the ratio of cells to 50:1, significant target-cell destruction by thymocytes occurred in two of six instances; 2% isotope release was detected. At this increased ratio, hepatocytes produced significant release of ^{51}Cr in but one of five instances, with 3% isotope release.

Lymphoid cells and target cells without PHA failed to produce significant release of ^{51}Cr, except in a few isolated instances.

DISCUSSION

We have shown a striking difference in the ability of cells from various fetal lymphoid organs to be stimulated to synthesize larger amounts of DNA and to mediate target-cell destruction. The discordance in these cell-mediated phenomena was most conspicuous in lymphoid cells from thymus and from the bone marrow.

Fetal thymocytes responded to PHA stimulation by incorporating thymidine into DNA, which is in agreement with existing data (10–12). However, there is considerable disagreement regarding the ability of PHA to induce DNA synthesis in thymocytes from various adult animal species (16). In the mouse, a cortisone-resistant, thymus-dependent population responsive to PHA has been clearly identified (17–19). In man, several investigators have found low levels of response (20, 21), and others have postulated two cell populations, the smaller of which is PHA-responsive (22). The failure to detect significant levels of increased DNA synthesis in earlier studies may reflect the narrow dose range of PHA used. On the other hand, the fetal thymus may contain a relatively larger proportion of PHA-responsive cells than does the adult organ.

Although the precise significance of PHA-induced synthesis of DNA is unknown, recent studies suggest that the response is specific for thymus-derived lymphocytes (6) and that the subpopulation of cortisone-resistant, PHA-responsive thymocytes in the mouse is fully immunocompetent (19). Our findings of PHA-responsive cells in the 14- to 18-week human fetal thymus suggest that some degree of immune competence exists in this lymphoid organ by the end of the first half of gestation.

While PHA induced significant synthesis of DNA in fetal thymocytes after 72 hr of culture, no appreciable target-cell destruction by these cells was produced during the first 20 hr of culture. Holm and Perlmann (3) were unable to induce

TABLE 1. *Toxicity of fetal lymphoid cells for chicken erythrocytes**

Organ	Chicken erythrocytes + cells: cpm released (cpm)	Chicken erythrocytes + cells: cpm released (%)	Chicken erythrocytes + cells + PHA: cpm released (cpm)	Chicken erythrocytes + cells + PHA: cpm released (%)
Thymus	34	0.7	29	0
Spleen	20	0	70	5.4
Blood	38	1.1	41	0.2
Liver	27	0	26	0
Bone marrow	28	0	152	19
Adult blood	30	0	161	21

* Results are the mean of triplicate determinations in an 18-week fetus. Background controls for this experiment, in cpm of ^{51}Cr released, were: chicken erythrocytes, 31; chicken erythrocytes, + PHA, 40; total cpm in pellet of chicken erythrocytes, 587.

target-cell destruction of either allogeneic or xenogeneic cells by PHA-stimulated rat thymocytes. Holm (21) has demonstrated that adult human thymocytes were unable to destroy Chang cells *in vitro* but, nevertheless, were able to respond to PHA by increased DNA synthesis. MacLennan and Harding (23) have clearly deomonstrated that rat thymocytes were not cytotoxic for antibody-coated Chang cells, but these thymocytes synthesized DNA normally in response to PHA. A similar dichotomy in the cytotoxicity of human thymocytes for Chang cells has been demonstrated recently by Hardy *et al.* (24). Harding *et al.* (25) have shown that PHA-induced DNA synthesis in peripheral lymphoid cells is thymus-dependent, while cytotoxicity of these cells for antibody-sensitized Chang cells is thymus-independent. Recently, a striking dissociation of DNA synthesis and cytotoxicity has been demonstrated in lymphocytes treated with concanavalin A (26).

Using a different lymphocyte–target cell system, Blomgren *et al.* (18) showed that a small subpopulation of cortisone-resistant, PHA-responsive rat thymocytes could specifically kill allogeneic target cells to which the animals had been sensitized. However, his study is not directly comparable to ours, because we used xenogeneic target cells and PHA-induced cytotoxicity with unsensitized lymphocytes. Our results do not exclude the presence of a small population of cytotoxic thymocytes. Our method may not be sensitive enough to detect a small subpopulation; it is also possible that our fetal specimens had not reached the developmental age at which such a subpopulation would appear.

Lymphoid cells from fetal bone marrow uniformly produced significant target-cell damage but failed to synthesize DNA in response to PHA. Our results suggest that lymphoid cells from bone marrow are the source of cells responsible for this pattern of response *in vitro*. This suggests that lymphocytotoxicity is primarily a bone marrow-derived and thymus-independent function. However, our study does not provide any direct evidence for the migration of bone marrow-derived or thymus-derived populations into peripheral lymphoid organs where both immunologic functions are expressed.

Recently, antibody-dependent, lymphocyte-mediated cytotoxicity has been demonstrated to be independent of thymus-derived lymphocytes in nonsensitized mice (27). These experiments also provide evidence that bone marrow-dependent (B) cells mediate destruction of antibody-coated target cells. The further discovery that both thymus (T)- and bone marrow (B)-dependent lymphocytes contain approximately equal numbers of binding sites for PHA (28), combined with the knowledge that chicken erythrocytes also contain PHA receptors, suggests that target-cell destruction by bone-marrow lymphoid cells in our preparation is a consequence of binding of marrow cells to the target.

No previous studies in guinea pigs (29), rats (23), anemic adults (30), or human fetuses (12) have shown PHA-induced DNA synthesis in bone-marrow cells. The existence of a minor subpopulation of apparently thymus-independent (19) lymphoid cells that responds to PHA by synthesis of DNA can be revealed in mouse bone marrow after treatment with cortisone (31). The presence of bone-marrow lymphocytes in the rat that are cytotoxic for antibody-coated Chang cells has been reported (23); however, we know of no previous reports that demonstrate this function in any human bone-marrow cultures.

TABLE 2. *Statistically significant release of ^{51}Cr stimulated by PHA in various fetal lymphoid organs*

Organ	Samples with significant release of ^{51}Cr*	Average ^{51}Cr release where significant (%)
Thymus	1 of 6	1.1
Spleen	3 of 4	3.8
Blood	3 of 4	14.6
Liver	1 of 6	1.5
Bone marrow	5 of 6	21.0
Adult blood	6 of 7	17.5

* Mean of triplicate experimental samples compared by Student's *t*-test to a mean of triplicate control samples ($P < 0.05$).

Despite the limitations inherent in the study of the immune response in humans, considerable information regarding the immune competence and specialization of various human fetal lymphoid organs has been gleaned from the present investigation. One may speculate that the precisely converse responses of the thymocyte and the bone-marrow cell provide evidence for thymus-dependent and bone marrow-dependent populations, and that the intermediate responses of lymphoid cells present in the peripheral blood and spleen† could be explained by the proposal that a mixture of the two independent cell populations exists.

Without the use of thymectomy and adoptive-transfer experiments, we cannot at this time conclude that the populations found in fetal thymus and bone marrow are equivalent to the thymus-dependent (T) cell and bone marrow-dependent (B) cell systems that have been identified in other animal species (32). Based upon the striking dichotomy of PHA-dependent responses of the fetal thymocytes and bone marrow cells, it is our conclusion that PHA-dependent cytotoxicity and DNA synthesis are functions of different lymphoid cell populations during human embryonic development.

We thank Ms. Joanne Rush and Ms. Janice Perlman for their expert technical assistance. D. P. S. is a USPHS postdoctoral trainee supported by Training Grant HE-05677. This work was supported in part by USPHS Grants HD-05894 and HD-03939.

1. Naspitz, Ch. K. & Richter, M. (1968) "The action of phytohemagglutinin *in vivo* and *in vitro*, a review," *Progr. Allergy* **12**, 1–85.
2. Holm G, Perlmann, P. & Werner, B. (1964) "Phytohaemagglutinin-induced cytotoxic action of normal lymphoid cells on cells in tissue culture," *Nature* **203**, 841–843.
3. Holm, G. & Perlmann, P. (1965) "Phytohaemagglutinin-induced cytotoxic action of unsensitized immunologically competent cells on allogeneic and xenogeneic tissue culture cells," *Nature* **207**, 818–821.
4. Lundgren, G. & Möller, G. (1969) "Non-specific induction of cytotoxicity in normal human lymphocytes *in vitro*: studies of mechanism and specificity of the reaction," *Clin. Exp. Immunol.* **4**, 435–452.

† Although both cell populations may arise in the spleen and populate the thymus and bone marrow, the appearance of both the proliferative response in the thymus and the cytotoxic potential of bone-marrow cells before a spleen was present make this alternative unlikely.

5. Holm, G. & Perlmann, P. (1967) "Cytotoxic potential of stimulated human lymphocytes," *J. Exp. Med.* **125**, 721–736.
6. Janossy, G. & Greaves, M. P. (1971) "Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens," *Clin. Exp. Immunol.* **9**, 483–498.
7. Doenhoff, M. J., Davies, A. J. S., Leuchars, E. & Wallis, W. (1970) "The thymus and circulating lymphocytes of mice," *Proc. Roy. Soc. Ser. B*, **176**, 69–85.
8. Oppenheim, J. J. (1968) "Relationship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man," *Fed. Proc.* **27**, 21–28.
9. Perlmann, P. & Holm, G. (1969) "Cytotoxic effects of lymphoid cells *in vitro*," *Advan. Immunol.* **11**, 117–193.
10. Pegrum, G. D., Ready, D. & Thompson, E. (1968) "The effect of phytohaemagglutinin on human foetal cells grown in culture," *Brit. J. Haematol.* **15**, 371–376.
11. Papiernik, M. (1970) "Correlation of lymphocyte transformation and morphology in the human fetal thymus," *Blood J. Hematol.* **36**, 470–479.
12. Kay, H. E. M., Doe, J. & Hockley, A. (1970) "Response of human foetal thymocytes to phytohaemagglutinin (PHA)," *Immunology* **18**, 393–396.
13. Böyum, A. (1968) "Separation of leucocytes from blood and bone marrow: with special reference to factors which influence and modify sedimentation properties of hematopoietic cells," *Scand. J. Clin. Lab. Invest. Suppl.* **97**, 1–109.
14. Hartzman, R. J., Segall, M., Bach, M. L. & Bach, F. H. (1971) "Histocompatibility matching. VI. Miniaturization of the mixed leukocyte culture test: a preliminary report," *Transplantation* **11**, 268–273.
15. Perlmann, P., Perlmann, H. & Holm, G. (1968) "Cytotoxic action of stimulated lymphocytes on allogenic and autologous erythrocytes," *Science* **160**, 306–309.
16. Forsdyke, D. R. (1969) "Impaired activation of thymus lymphocytes by phytohemagglutinin," *J. Immunol.* **103**, 818–823.
17. Blomgren, H. & Svedmyr, E. (1971) "Evidence for thymic dependence of PHA-reactive cells in spleen and lymph nodes and independence in bone marrow," *J. Immunol.* **106**, 835–841.
18. Blomgren, H., Takasugi, M. & Friberg, S., Jr. (1970) "Specific cytotoxicity by sensitized mouse thymus cells on tissue culture target cells," *Cell. Immunol.* **1**, 619–631.
19. Blomgren, H. & Svedmyr, E. (1971) "*In vitro* stimulation of mouse thymus cells by PHA and allogeneic cells," *Cell. Immunol.* **2**, 285–299.
20. Winkelstein, A. & Craddock, C. G. (1965) "Comparative response of normal human thymus and lymph node cells to phytohemagglutinin in culture," *Blood J. Hematol.* **26**, 876–877.
21. Holm, G. (1967) "Lack of cytotoxicity by human thymocytes *in vitro*," *Scand. J. Haematol.* **4**, 230–240.
22. Claman, H. N. (1966) "Human thymus cell cultures—evidence for two functional populations," *Proc. Soc. Exp. Biol. Med.* **121**, 236–240.
23. MacLennan, I. C. M. & Harding, B. (1970) "Failure of certain cytotoxic lymphocytes to respond mitotically to phytohaemagglutinin," *Nature* **227**, 1246–1248.
24. Hardy, D. A., Ling, N. R., Wallin, J. & Aviet, T. (1970) "Destruction of lymphoid cells by activated human lymphocytes," *Nature* **227**, 723–725.
25. Harding, B., Pudifin, D. J., Gotch, F. & MacLennan, I. C. M. (1971) "Cytotoxic lymphocytes from rats depleted of thymus processed cells," *Nature* **232**, 80–81.
26. Perlmann, P., Nilsson, H. & Leon, M. A. (1970) "Inhibition of cytotoxicity of lymphocytes by concanavalin A *in vitro*," *Science* **168**, 1112–1115.
27. Van Boxel, J. A., Stobo, J. D., Paul, W.E. & Green, I. (1972) "Antibody-dependent lymphoid-cell mediated cytotoxicity: No requirement for thymus-derived lymphocytes," *Science* **175**, 194–196.
28. Stobo, J. D., Rosenthal, A. S. & Paul, W. E. (1972) "Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of concanavalin A and phytohemagglutinin," *J. Immunol.* **108**, 1–17.
29. Winkelstein. A. (1971) "Failure of lymphocytes from guinea pig bone marrow to respond to phytohemagglutinin," *Proc. Soc. Exp. Biol. Med.* **136**, 578–583.
30. Pegrum, G. D., Ready, D. & Thompson, E. (1968) "The *in vitro* effect of phytohaemagglutinin on separated human bone marrow cells," *Brit. J. Haematol.* **15**, 377–380.
31. Levine, M. A. & Claman, H. N. (1970) "Bone marrow and spleen: dissociation of immunologic properties by cortisone," *Science* **167**, 1515–1516.
32. Playfair, J. H. L. (1971) "Cell cooperation in the immune response," *Clin. Exp. Immunol.* **8**, 839–856.