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Ontogeny of Human Cell-Mediated Immunity: Age-Related Variation of In Vitro Infantile Lymphocyte Transformation

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Thymidine uptake of unstimulated and of phytohemagglutinin (PHA)-, streptokinase-streptodornase (SK-SD)- and candida extract (candida)-stimulated lymphocytes of normal infants less than 20 months old was evaluated. Thymidine uptake of unstimulated and PHA-stimulated young infants' lymphocytes was avid, resembling that of newborn cells. Over periods of weeks to months, infantile lymphocytes demonstrated transition in unstimulated and PHA-induced thymidine uptake from response patterns like cord blood cells to ones more characteristic of adult lymphocytes. Candida- and SK-SD-induced thymidine uptake of lymphocytes from very young infants was likewise apparently quite avid, resembling cord blood cells. However, factoring out high unstimulated thymidine uptake by conversion of data to stimulation indexes clarified differing paces of acquisition of transformation responsiveness of the two naturally acquired infectious antigens. Specific lymphocyte reactivity to SK-SD was quite low in all groups of infants compared with candida-induced transformation, which in some infants acquired adult proportions.

The success with which a microbiologically sterile human newborn deals with a new environment rich in microorganisms attests to the adaptability of his immune system. The newborn infant, long thought of as "immunologically incompetent," is actually remarkably capable, although differing quantitatively and qualitatively in immune responsiveness from the adult (19, 21, 22). Maternally acquired immunoglobulins are inadequate to deal with every infantile infectious antigenic challenge. Since maternal cell-mediated immunity does not appear to be passively transferred, active primary cell-mediated immunity would appear to provide a major bulwark against neonatal infection (7). The experiments reported herein concern one parameter reflecting the ontogeny of human cell-mediated immune response. Transformation of lymphocytes from normal infants has been assessed and compared with that of lymphocytes from newborns (cord blood) and adults. During a period of several months a transition was demonstrated in thymidine uptake reactivity from responses like those of cord blood cells toward patterns resembling those of adult lymphocytes.

MATERIALS AND METHODS

Heparinized (20 U/ml) umbilical vein venous blood (10 to 15 ml) was collected from eight neonates after normal full-term deliveries, and peripheral venous blood was collected from 37 healthy 1- to 20-month-old infants (5 to 10 ml) and eight healthy adults with a mean age of 35 years (30 ml). Blood was mixed with 4 volumes of 0.87% NH_4Cl solution to lyse erythrocytes. Leukocytes were sedimented by low-speed centrifugation (300 × *g*), washed twice, and resuspended in medium 199 (Flow Laboratories) containing glutamine (2 mM final concentration), penicillin (100 U/ml), and streptomycin (100 μg/ml). The lysis step was repeated once. Leukocytes suspended in approximately 3 ml of autologous plasma were adsorbed in medium-containing cotton wool-filled columns for 10 min at 37 C as previously described (1). Effluent nonadsorbent cells were sedimented by low-speed centrifugation, washed with medium, and resuspended in 20% serum-supplemented medium, and 1 million lymphocytes per 2 ml were distributed to culture tubes for incubation at 37 C in a humidified atmosphere of 5% CO_2 in air. Cultures also included 1 to 5% monocytes and numerous platelets. Cord blood leukocyte cultures also contained 0 to 13 lysis-resistant orthochromatic erythroblasts per 100 mononuclear cells. Stimulants for transformation were phytohemagglutinin (PHA-P; Difco; 0.1 ml of a 1:40 dilution of the reconstituted lyophilized mitogen, 70 μg/culture) and two soluble antigens, streptokinase-streptodornase (SK-SD; Lederle Laboratories; 0.1 ml containing 20 U of streptokinase and 5 U of streptodornase per culture), and candida extract (candida; Hollister Stier Laboratories; 0.1 ml containing 20 μg of protein per culture) (16). Dialysis and filtration of antigens was carried out as previously described (2). PHA-stimulated cells were cultured for 4 days; unstimulated,

SK-SD; and candia-stimulated cells were cultured for 6 days. During the final 18 h of incubation, 2.0 μCi of tritiated thymidine (New England Nuclear Corp., specific activity, 6.7 Ci/mmol) was added. Harvesting procedures and liquid scintillation counting of the trichloroacetic acid-precipitable residue was performed by previously reported methods (1). Data are reported in disintegrations per minute. Duplicate cultures were run and the means were recorded. Student's t test was applied for statistical analysis of group differences. Only differences with $P < 0.01$ were considered to be significant.

RESULTS

Thymidine uptake of unstimulated lymphocyte cultures is indicated in Fig. 1. Lymphocytes from cord blood (mean, 11,118 ± 5,642 standard deviation [SD]) and infants less than 3 months old (mean, 3,262 ± 551 SD) spontaneously transformed more than those from adults (mean, 1,081 ± 546 SD). A consistent trend downward in spontaneous in vitro blastogenesis was observed during the first year of life, but the degree of transformation of unstimulated lymphocytes from infants older than 3 months did not differ significantly from adult cells. Observed high unstimulated lymphocyte counts in cord blood might have resulted in part from thymidine uptake of orthochromatic erythroblasts (nucleated erythrocytes), which were frequently observed in cord blood lymphocyte specimens even after purification procedures. However, rank correlation coefficient testing (11) revealed no relationship between number of nucleated erythrocytes and thymidine uptake, indicating a minor role for nucleated erythrocytes in thymidine uptake of our unstimulated lymphocyte cultures. Effects of newborn serum, which by virtue of α^1-fetoglycoprotein content may be suppressive to blastogenesis (4), or fetal bovine serum which may stimulate blastogenesis of otherwise unstimulated cultures (unpublished observation), were avoided by use of nontoxic, pooled, heat-inactivated human serum for medium supplementation.

PHA was used in a concentration that in our laboratory results in maximal stimulation of adult lymphocytes cultured in 20% serum. PHA-stimulated cord blood lymphocytes (Fig. 2) incorporated over twice as much of thymidine (mean, 553,695 ± 244,666 SD) as did adult lymphocytes (226,095 ± 100,100 SD). A trend somewhat like that of unstimulated cells toward diminishing thymidine uptake was noted in PHA-lymphocyte cultures from infants less than 1 year old. Lymphocytes from all groups of infants less than 10 months old demonstrated significantly higher transformation values

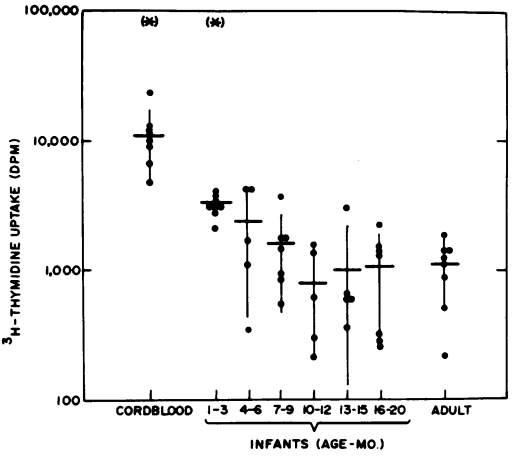

FIG. 1. *Tritiated thymidine uptake of unstimulated lymphocytes. Data points indicate values for individual subjects. In this and Fig. 2–4, values are grouped according to age on the horizontal axis. Arithmetic means are indicated by the horizontal bars and standard deviation by the vertical lines. Groups differing significantly in thymidine uptake from adults ($P < 0.01$) are indicated by an asterisk.*

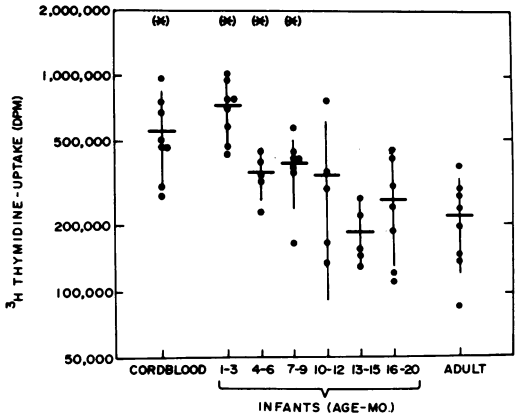

FIG. 2. *Tritiated thymidine uptake of PHA-stimulated cord blood, infantile, and adult human lymphocytes. Seventy micrograms of PHA-P was added per culture.*

than did lymphocytes obtained from adults.

Antigen-induced transformation of cord blood and infantile lymphocytes yielded a more complicated pattern of age-related results. SK-SD (Fig. 3) yielded what appeared to be a biphasic pattern of transformation relative to age. Lymphocytes from cord blood and from all groups of infants were stimulated significantly less by SK-SD than were adult lymphocytes. SK-SD-stimulated cord blood lymphocytes incorporated significantly more thymidine than did any group of infantile lymphocytes, and

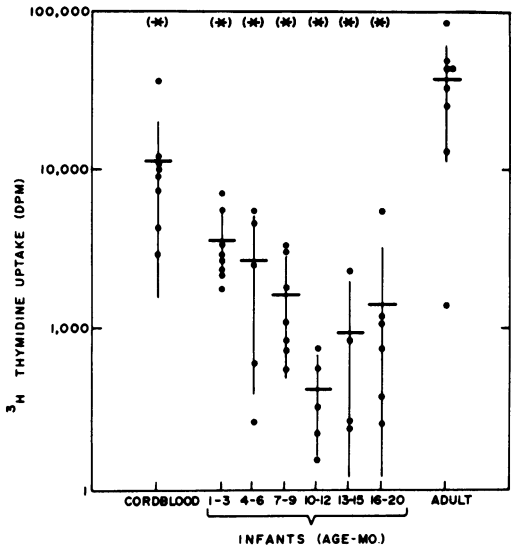

FIG. 3. *Tritiated thymidine uptake of SK-SD-stimulated cord blood, infantile, and adult lymphocytes. Twenty units of streptokinase and 5 U of streptodornase were added per culture.*

those from cord blood and from very young infants incorporated more isotope than did lymphocytes from older infants (cord versus > 10 months, $t = 4.1$, $P < 0.001$; 1 to 3 months versus >10 months $t = 3.9$, $P < 0.001$). SK-SD induced blastogenesis declined (cord blood mean, 11,695 ± 10,200 SD) to minimal levels in the 10- to 12-month age group (mean, 408 ± 253 SD). Adult lymphocytes all reacted to SK-SD (mean, 36,999 ± 25,589 SD).

Thymidine uptake induced by candida extract (Fig. 4) followed a somewhat similar pattern to that of SK-SD, with lowest uptake in cultures from lymphocytes of infants aged 10 to 12 months (mean, 2,331 ± 2,354 SD) as compared with newborns (mean, 10,937 ± 7,007 SD) and adults (mean, 34,920 ± 30,200 SD).

With correction of data to account for concurrently declining unstimulated, control culture values by expressing transformation results as a ratio of experimentally stimulated (E) to control (C) cultures, the stimulation index (E/C) nullified the apparent paradoxical decline in antigen-induced blastogenesis up to 1 year of age. SK-SD stimulation indexes are indicated in Fig. 5. Significant stimulation indexes for cord blood lymphocytes or infantile lymphocytes occured only once, in a 15-month-old female with a stimulation index of 4.3.

In contrast to SK-SD effects, significant (E/C > 2 arbitrarily assigned significance) candida-induced transformation (Fig. 6) occurred in lymphocytes from 15 to 37 infants tested. Positive candida-induced stimulation indexes were present in all lymphocyte cultures from infants older than 1 year. In six infants stimulation indexes were in the range of those of healthy adults studied.

DISCUSSION

Functional differences between adult human and fetal lymphocytes have been extensively

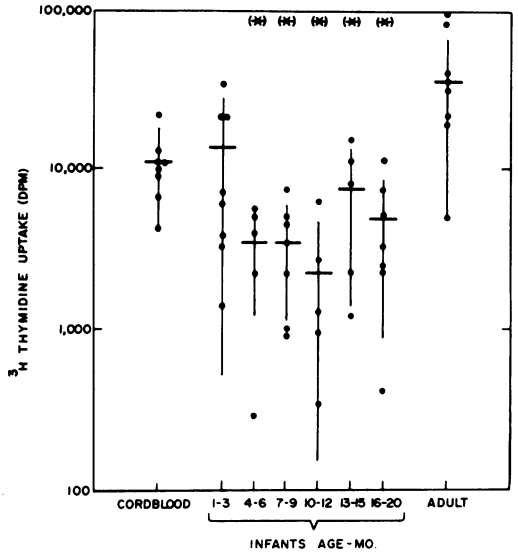

FIG. 4. *Tritiated thymidine uptake of candida extract-stimulated cord blood, infantile, and adult lymphocytes. Candia containing 20 μg of protein was added per culture.*

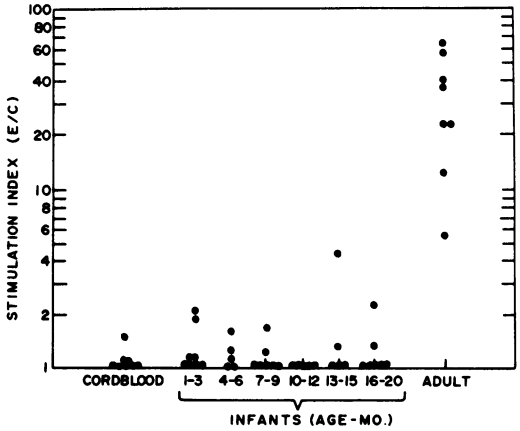

FIG. 5. *SK-SD-induced transformation of cord blood, infantile, and adult lymphocytes. The vertical logarithmic axis indicates the stimulation index. Indexes of less than one were assigned a value of one. Data points indicate values calculated from means of duplicate tests for individual subjects.*

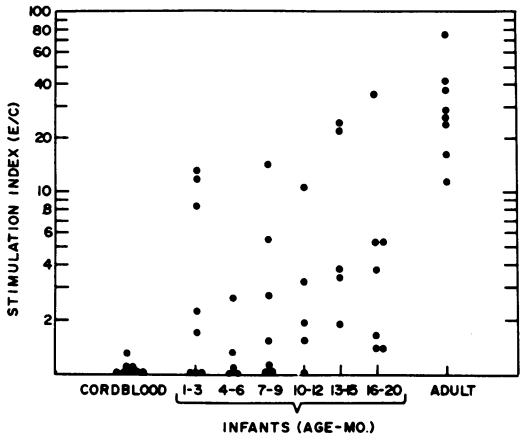

FIG. 6. *Candida extract-induced transformation of cord blood, infantile, and adult lymphocytes. Stimulation indexes are indicated.*

investigated. Umbilical cord venous lymphocytes in culture undergo a greater degree of "spontaneous transformation" than do adult lymphocytes (17). Acceleration of spontaneous transformation, as judged by morphology, apparently occurs by 33 weeks of gestation (10). Comparisons of spontaneous blastogenesis of cord and adult blood lymphocytes by a number of investigators have indicated significant differences. Our finding of greater thymidine uptake of unstimulated cord blood lymphocytes than adult lymphocytes confirms the observations of several investigators (6, 13, 17).

Our data indicate that avid thymidine uptake by unstimulated lymphocytes extends beyond the day of birth into the early weeks of life. Lymphocytes from infants less than 3 months old incorporated significantly more thymidine (mean, 3,262 dPM ± 566 SD) than did those from adults (mean, 1,081 dPM ± 550 SD). Since effects of maternal-cell admixture and fetal erythroblasts would have ended by this age, we conclude that infantile lymphocytes react much as cord blood lymphocytes in incorporating more thymidine than adult cells. Increased spontaneous thymidine uptake of infants as compared with adults was confined to the group 3 months old and younger. High spontaneous incorporation of thymidine by cord blood and young infantile lymphocytes could be related to deoxyribonucleic acid synthesis of either T or B cells.

Further evidence that infantile lymphocytes undergo maturation during early months of postnatal life has been provided by our PHA stimulation studies. Upon PHA stimulation, lymphocyte thymidine uptake of a degree greater than that of adult cells was found to persist from birth until up to 9 months of age. Blastogenesis in response to PHA, predominantly a T cell response (9), has been correlated with developing cell-mediated immune competence. Events relating to ontogeny of human fetal cell-mediated immunity including lymphocyte-PHA responsiveness have recently been reviewed (23). As early as 15 to 17 weeks of gestation, fetal lymphocyte transformation occurs in response to PHA (10, 15). Fetal lymph node (10) and thymic (24) lymphoid cells incorporate more thymidine upon PHA stimulation than do splenic cells.

Cord blood PHA-lymphocyte responsiveness compared with adult reactivity has been subjected to extensive evaluation with conflicting results, probably resulting from methodological differences (23). Assays of PHA-induced lymphocyte transformation of cord blood lymphocytes have purported to show reactivity greater than (5, 6, 8), equal to (5, 10, 12), and less than (4, 10) that of adult lymphocytes. Both high (5) and low (6) concentrations of PHA have been shown to accentuate differences in transformation between cord blood and adult lymphocytes. Though extensive PHA dose-response testing of cord blood and infantile cells was not performed, the concentration used in the current study optimally stimulates adult lymphocytes and appears to satisfactorily differentiate adult, neonatal, and infantile cells.

Investigations revealing greater cord blood PHA-induced transformation (including the present one) have utilized tritiated thymidine uptake as indicative of blastogenesis. A dichotomy may exist between isotope uptake and morphological transformation of lymphocytes (25). Our studies were performed without comparison of morphological transformation to thymidine uptake, and further investigation is needed to ascertain whether elevated thymidine uptake of infantile lymphocytes is associated with morphological blastogenesis. Regardless, infantile lymphocytes appear to undergo a functional maturation during the early months of postnatal life as indicated by the parameter of PHA-induced thymidine uptake.

Although responding to PHA by thymidine uptake, fetal lymphocytes fail to mediate PHA-dependent nonspecific cytotoxicity. This dichotomy has been interpreted as representing an immunological developmental stage (24). Lymphocyte proliferation without lymphokine production has been termed "dissociated lymphocyte function" (18). Recently, dissociation of PHA-induced blastogenesis and cytotoxicity has been ascribed to cord blood lymphocytes. Newborn lymphocytes have been characterized

by PHA-induced thymidine uptake greater than that of adult cells and cytotoxic reactivity strikingly less than that of adult lymphocytes (18, 19). Our results relating to avid thymidine uptake by lymphocytes of young infants suggest that their cytotoxic activity should be assessed and may be low in relation to blastogenesis.

Antigen-induced lymphocyte transformation of newborn lymphocytes has been much less extensively evaluated than mitogen-induced blastogensis. Two weeks after immunization with typhoid vaccine, cultured lymphocytes of newborn infants respond specifically upon exposure to salmonella antigen with blastogenesis comparable in degree to that of lymphocytes obtained from adults (12). Lymphocytes from unimmunized neonates fail to transform when cultured with tetanus and diphtheria toxoids, or complexes of these antigens with their respective antitoxins, further supporting specificity of neonatal lymphocyte transformation (13). However, significant transformation of cord blood lymphocytes has been reported in response to two microbial antigens (pneumococcus type I extract and streptolysin O) to which neonates would not have been expected to have had exposure (14).

Specific reactivity to the two soluble natural microbial antigens we studied was clarified when data were converted to stimulation indexes. Lack of appreciable reactivity to SK-SD, a potent blastogenic antigen for adult lymphocytes (26), except in one infant studied, probably resulted from lack of sensitizing infections with *Streptococcus pyogenes* in utero or during the first 20 months of life (3). Infants less than 2 years old lack delayed-type skin responsiveness to streptococcal nucleoprotein, whereas children over 6 years old usually react to that antigen (21). In vitro transformation of cord blood cells exposed to streptolysin O but not to streptococcal cell wall extract (23) could indicate a mitogenic effect of streptolysin O. The lack of cellular immunological responsiveness of young infants to most streptococcal products not only reflects lack of exposure, but probably in part determines the peculiar differences of infantile and adult inflammatory response to streptococcal infection.

Candida albicans extract-induced positive stimulation indexes occurred by a few weeks of age in approximately half of the infants tested, probably reflecting sensitization to candida from neonatal colonization. Some degree of candida-induced blastogenesis existed in all lymphocyte cultures from infants older than 1 year comparing favorably with 80% delayed skin test reactivity to candida extract in infants 7 to 12 months old (20). Stimulation indexes of a number of infants in the current study were comparable to normal adult levels, often exceeding values for hospitalized adults reported previously (2). While undergoing functional maturation during early months of life, infantile lymphocytes apparently transform in a like manner to adult cells when in vivo sensitization to the antigen has occurred. A gradually increasing stimulation index characterizing the normal infantile lymphocyte response to *C. albicans* antigen provides an example of the pace of acquisition and magnitude of specific cell-mediated immunity to a ubiquitous infection.

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LITERATURE CITED

1. **Alford, R. H.** 1970. Metal cation requirements for phytohemagglutinin-induced transformation of human peripheral blood lymphocytes. J. Immunol. **104**:698–703.
2. **Alford, R. H.** 1973. Transformation of lymphocytes of normal and hospitalized adults by Candida albicans extract. Proc. Soc. Exp. Med. Biol. **144**:826–829.
3. **Alpert, J. J., M. R. Pickering, and R. J. Warren.** 1966. Failure to isolate streptococci from children under the age of 3 years with exudative tonsillitis. Pediatrics **38**:663–666.
4. **Ayoub, J., and S. Kasakura.** 1971. *In vitro* response of foetal lymphocytes to PHA, and a plasma factor which suppresses the PHA response of adult lymphocytes. Clin. Exp. Immunol. **8**:427–434.
5. **Campbell, A. C., C. Waller, J. Wood, A. Anysley-Green, and V. Yu.** 1974. Lymphocyte subpopulations in the blood of newborn infants. Clin. Exp. Immunol. **18**:469–482.
6. **Carr, M. C., D. P. Stites, and H. H. Fudenberg.** 1972. Cellular immune aspects of the human fetal-maternal relationship. I. *In vitro* response of cord blood lymphocytes to phytohemagglutinin. Cell Immunol. **5**:21–29.
7. **Editorial.** 1969. Lancet **2**:253–255.
8. **Eife, R. F., G. Eife, C. S. August, W. L. Kuhre, and J. Staehr-Johansen.** 1974. Lymphotoxin production and blast cell transformation by cord blood lymphocytes: dissociated lymphocyte function in newborn infants. Cell. Immunol. **14**:435–442.
9. **Janossy, G., and M. P. Greaves.** 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. Clin. Exp. Immunol. **9**:483–498.
10. **Jones, W. R.** 1969. In vitro transformation of fetal lymphocytes. Am. J. Obstet. Gynecol. **104**:586–592.
11. **Kendall, M. G.** 1970. Rank correlation methods, 4th ed. Chas. Griffin Co., London.
12. **Leiken, S., F. Mochir-Fatemi, and K. Park.** 1968. Blast transformation of lymphocytes from newborn human infants. J. Pediatr. **72**:510–517.
13. **Leiken, S., and J. J. Oppenheim.** 1971. Differences in transformation of adult and newborn lymphocytes by

antigen, antibody, and antigen-antibody complexes. Cell. Immunol. 1:468-475.

14. **Leiken, S., J. Whang-Peng, and J. J. Oppenheim.** 1970. In vitro transformation of human cord blood lymphocytes by antigens p. 389-402. *In* J. Harris (ed.), Proc. 5th Leukocyte Culture Conf. Appleton-Century-Crofts, New York.
15. **Lindahl-Kiessling, K., and J. A. Book.** 1964. Effects of phytohaemagglutinin on leucocytes. Lancet II:591.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
17. **Pulvertaft, R. J. V., and I. Pulvertaft.** 1966. Spontaneous "transformation" of lymphocytes from the umbilical-cord vein. Lancet 2:892-893.
18. **Rocklin, R. E., G. Reardon, A. Sheffer, W. H. Churchill, and J. R. David.** 1970. Dissociation between two *in vitro* correlates of delayed hypersensitivity: absence of migration inhibitory factor (MIF) in the presence of antigen-induced incorporation of ^{3}H-thymidine, p. 639-648 *In* J. Harris (ed.), Proc. 5th Leukocyte Culture Conf. Appleton-Century-Crofts, New York.
19. **Rosen, F. S., and C. A. Janeway.** 1964. Immunological competence of the newborn infant. Pediatrics 33:159-160.
20. **Shannon, D. C., G. Johnson, F. S. Rosen, and K. F. Austen.** 1966. Cellular reactivity to *Candida albicans* antigen. N. Engl. J. Med. 275:690-693.
21. **Smith, R. T.** 1960. Immunity in infancy. Pediatr. Clin. North Am. 7:269-293.
22. **Smith, R. T., and D. C. Eitzman.** 1964. The development of the immune response. Characterization of the response of the human infant and adult to immunization with salmonella vaccines. Pediatrics 33:163-183.
23. **Stites, D. P., J. Caldwell, M. C. Carr, and H. H. Fudenberg.** 1975. Ontogeny of immunity in humans. Clin. Immunol. Immunopathol. 4:519-527.
24. **Stites, D. P., M. D. Carr, and H. H. Fundenberg.** 1972. Development of cellular immunity in the human fetus: dichotomy of proliferative and cytotoxic responses of lymphoid cells to phytohemagglutinin. Proc. Natl. Acad. Sci. U.S.A. 69:1440-1444.
25. **Sutherland, R. M., W. R. Inch, and J. A. McCredie.** 1971. Phytohemagglutinin (PHA)-induced transformation of lymphocytes for patients with cancer. Cancer 27:574-578.
26. **Taranta, A., G. Cuppari, and F. Quagliata.** 1968. High lymphocyte transformation with nonhaemolytic streptococcal product. Nature (London) 219:757-758.