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FAMILIAL THYMIC APLASIA

Attempted Reconstitution with Fetal Thymus in a Millipore Diffusion Chamber

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Abstract A 10-week-old female infant with familial congenital thymic aplasia without delayed hypersensitivity to common skin-test antigens underwent fetal-thymus implantation. Six hours after the implantation of a fetal thymus enclosed in a Millipore chamber phytohemagglutinin responsiveness was demonstrable in the patient's peripheral lymphocytes. The infant's death of aspiration pneumonia nine days after implantation did not allow evaluation of the extent of the immunologic reconstitution. Thymic-cell immunologic function can be induced in man with fetal-thymus humoral factors.

CONGENITAL absence of the thymus and parathyroid glands was first fully appreciated as a distinct clinical syndrome in 1965.[1] The apparent etiology is a failure of embryonic development of the third and fourth pharyngeal pouches, the anlage of the thymus and parathyroid glands. No hereditary predisposition has previously been described. Affected infants become symptomatic shortly after birth, exhibiting hypocalcemic tetany or convulsions and severe retardation of growth and development. Later in infancy they manifest an increased clinical severity with infectious agents that are normally handled through cell-mediated responses (e.g., monilia). Therefore, early recognition of the disorder is imperative since immunization with live vaccines may be fatal.

Recent reports demonstrate that the implantation of fetal thymus tissue into these patients restores cell-mediated immune function[2-4]; fetal tissue was used to minimize the risk of a graft-versus-host reaction. The use of thymus tissue in Millipore diffusion chambers or purified thymic extract for thymectomized animals has resulted in at least partial reconstitution.[5-7] Such methods decrease greatly the risk of the graft-versus-host reaction; application to treatment of immune deficiencies in the clinical setting is therefore apparent.

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CASE REPORT

A Caucasian female infant weighed 3.3 kg. at birth; the initial physical examination was described as unremarkable. The 29-year-old mother's only other pregnancy was by a previous marriage and resulted in the birth of a male infant who died at 4 months of age.

The patient's 3-day nursery course was uneventful, but the mother noted difficulty with feeding and frequent "spitting-up" shortly after returning home. At 5 to 6 days of age "twisting and jerking" motions of the extremities were observed. At 12 days of age the patient had 2 generalized seizures and was subsequently referred to Georgetown University Hospital. Physical examination demonstrated a lethargic infant with generalized depression of neurologic function, hypertelorism, and slightly low-set ears without malformation of the pinnae. A thymic shadow was not observed on routine chest roentgenograms. Other abnormal laboratory values included a serum calcium of 6.2 to 7.4 and a phosphorus of 5.5 to 8.0 mg per 100 ml.

Initial immunologic studies revealed white-cell counts of 13,500 to 17,600, absolute neutrophil counts of 1500 to 3520 and absolute lymphocyte counts of 4050 to 6160 per cubic millimeter. Total complement activity and β1c-1a globulin concentrations were within normal limits. Serial quantitative immunoglobulin determinations at 3, 6 and 12 weeks were within normal limits for age. A maximum flagellar (H) agglutination titer increased from less than 1:2 to 1:80 14 days after stimulation with typhoid vaccine,* agglutinins to somatic (O) antigen were not detected. An excisional biopsy of right inguinal lymph nodes was obtained 12 days after intradermal injection of 0.4 ml of typhoid vaccine into the right medial thigh. Attempts to stimulate peripheral blood lymphocytes in vitro with phytohemagglutinin (PHA) and pokeweed mitogen elicited almost no response (Table 1). Intradermal injection of 1 μg and 2 μg of PHA resulted in no induration or erythema at the site of injection.

The hospital course was relentlessly downhill. Hypocalcemia was initially treated with intramuscular parathyroid hormone and subsequently with oral vitamin D. However, wide fluctuations of serum calcium levels made management difficult. A peripheral blood eosinophil count of 41 to 49 per cent was noted. Growth remained severely retarded, and at 10 weeks the weight was 3.2 kg; she refused

*Typhoid vaccine, U.S.P., Eli Lilly Co., Indianapolis, Ind.

Table 1. Uptake of ^{3}H-Thymidine by Lymphocytes Stimulated with PHA and Pokeweed before and after Fetal-Thymus Graft in a Patient with Congenital Aplasia of the Thymus.

Study Period	Counts/Min/4 × 10^5 Viable Lymphocytes						
	Control*	1% PHA-P	0.1% PHA-P	0.01% PHA-P	4% Pokeweed	1% Pokeweed	0.1% Pokeweed
Before graft	1,450	2,497	2,345	1,773	5,867	3,056	1,704
After graft	2,125	81,105	125,275	50,667	–	–	–
Control donor	1,038	123,532	156,234	113,184	50,333	77,476	50,105

*Control wells contain lymphocytes but no stimulating agents

feedings and rapidly became oliguric. A rising BUN and serum sodium was treated with fluid therapy and salt restriction. Shortly thereafter, fetal thymus in a Millipore chamber was implanted under the right rectus abdominis sheath. In vitro PHA-stimulated lymphocyte transformation was then evaluated at periodic intervals. The patient continued to feed poorly, and on the 5th postoperative day vomited and aspirated. Bilateral pulmonary consolidation progressed in spite of aggressive management, and she died 9 days after implantation of the thymus tissue.

The half-brother of this patient, born 5 years previously, had a clinical course quite similar to hers. He experienced generalized convulsions at 10 days of age, and diagnostic studies documented hypocalcemia. An eosinophil count of 16 to 30 per cent was also noted. Growth retardation was severe, and he died at the age of 4 months. At autopsy, performed at the National Institutes of Health, Bethesda, Maryland, neither the thymus nor the parathyroid glands could be identified grossly or in multiple sections of the mediastinal tissues and neck. Lymph nodes throughout the body were enlarged and contained many macrophages and eosinophils. The deep cortical zones were depleted of small lymphocytes. The outer cortex contained small lymphoid nodules with occasional germinal centers. The lymphoid tissue of the intestine and spleen were well developed except for some rarity of germinal centers. The pulmonary alveoli were filled with foamy granular material, and methenamine silver stains revealed massive *Pneumocystis carinii* infection.

The mother's medical history suggested partial thymus and parathyroid deficiencies. She had secondary vaccinia after routine vaccination at 2 years of age, and at 7, a 6-month hospitalization was required for "severe chicken pox." At 24 years of age carpopedal spasm, numbness of the hands and feet and leg cramping developed. Hypocalcemia responsive to parathyroid hormone was documented and she has been well controlled on oral calcium. Absolute lymphocyte counts ranged from 690 to 1050 and absolute eosinophil count 15 to 42 per cubic millimeter. In vitro lymphocyte stimulation with PHA and pokeweed demonstrated a quantitative deficiency, uptake of ^{3}H-thymidine being 15 to 50 per cent of control values; results were nearer normal at the lower concentrations of stimulants. Intradermal skin testing with monilia antigen, trichophyton, mumps, diphtheria-tetanus, streptokinase-streptodornase, PPD, histoplasmin, PHA and dinitrochlorobenzene (NDCB) were negative. Quantitative immunoglobulin determinations were within normal limits.

Methods

In vitro morphologic transformation of peripheral lymphocytes after stimulation with PHA was determined with the method of Moorehead,[8] which was modified to use 0.2 ml of blood for each determination.

After stimulation with PHA and pokeweed, incorporation of ^{3}H-thymidine into DNA was measured by methods previously described.[9] Lymphocytes were obtained from whole blood with the use of diatrizoate (Hypaque-Ficoll) separation gradient[10]; viability was determined with trypan blue, and the suspension was diluted to a concentration of 2×10^6 viable lymphocytes per milliliter. Triplicate samples were incubated with the stimulants in microtiter wells and harvested with a specially designed harvesting apparatus. Cultures with PHA were incubated for three days, and those with pokeweed for five days.

Peripheral blood lymphocytes isolated as described above were incubated for 45 minutes with ice-cold fluorescein-labeled antibodies to IgG, IgM, IgA and light chains,* and examined in wet-mount preparations to assay for surface immunoglobulins.[11,12]

Skin tests were injected intradermally in the forearm in 0.1-ml test doses and read at 48 and 72 hours. Less than 5 mm of induration or erythema (or both) was considered negative. DNCB contact sensitivity was tested by application of 0.1 ml of the acetone-diluted sensitizing and challenging material to an area on the forearm 2.5 cm in diameter until dry; after the challenging dose the area was observed at one, two, three, five, and seven and 14 days for erythema and vesiculation.

Quantitative immunoglobulin levels were determined by a standard radial diffusion technic.[13]

Biopsied lymph nodes were processed for light, immunofluorescence and electron microscopy by methods previously described.[14,15] Fluoresceinated antiserums for IgG, IgM, IgA, fibrinogen, and albumin (obtained from commercial sources) were applied to serial cryostat sections of lymph nodes for the identification of immunoglobulin-containing cells.

The thymus was obtained from a 13-week-female fetus (crown-to-rump length, 8.7 cm) after a hysterectomy therapeutic abortion performed elsewhere. The intact thymus was placed in a sterile 0.45 μ pore-size Millipore diffusion chamber,† which was kept in cooled tissue medium until implantation four hours later. The chamber was constructed from a methyl ester (Lucite) ring with an outside diameter of 14 mm, an inside diameter of 10 mm, and a depth of 2 mm; Millipore disks were glued to the outside surfaces of the ring. The chamber was implanted under the right rectus abdominis sheath.

Results

Quantitative immunoglobulin determinations at three, six and 12 weeks of age were normal for age, with maximum levels of IgA and IgM reaching 26 and 44 mg per 100 ml respectively by 12 weeks. An agglutination titer of 1:80 14 days after stimulation demonstrated a primary response to typhoid H antigen. Assay of peripheral blood lymphocytes for surface immunoglobulins revealed the following proportion of positive cells: IgG, 17.2 per cent; IgM, 5.7 per cent; IgA, 1.2 per cent; and light chain, 22 per cent. These values were within the normal control range. The patient's mother

*Kindly supplied by Dr. William Adler.

†Millipore Corporation, Bedford, Mass.

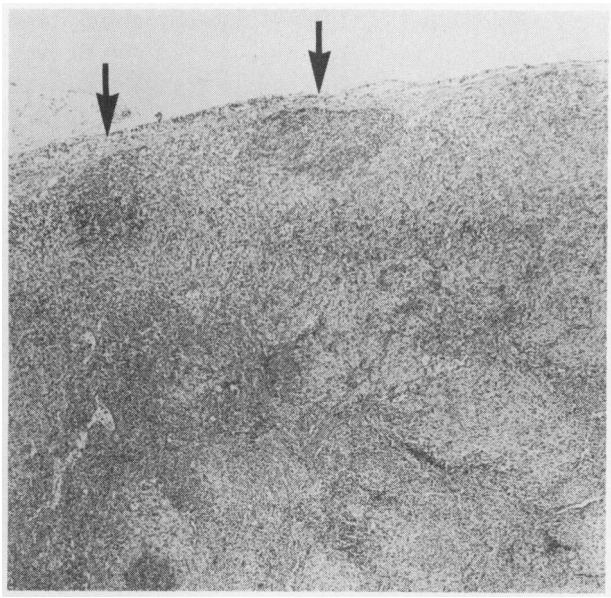

Figure 1. Inguinal Lymph Node of the Patient Excised 12 Days after Intradermal Injection of Typhoid Vaccine (Hematoxylin and Eosin Stain ×35).

Arrows indicate small lymphoid follicles without germinal centers in the outer cortex. The deep cortical zone is densely filled with numerous eosinophils and macrophages but few lymphocytes.

also had normal percentages of lymphocytes with membrane-bound immunoglobulins.

Microscopical sections of the inguinal-lymph-node excisional biopsy (Fig. 1) showed subcapsular follicles without germinal centers. The deep cortical zones contained only occasional lymphocytes and were populated by numerous macrophages and eosinophils. Immunofluorescence microscopy revealed occasional plasma cells containing IgG or IgM but none positive for IgA.

The response of the patient's lymphocytes to PHA as measured by uptake of ^{3}H-thymidine was negligible 17 days before implantation but a normal response was noted eight days after the implant (Table 1). Increased lymphocyte transformation after PHA stimulation was demonstrated as early as six hours after the implant (Table 2). PHA skin tests were negative before the implant but were not repeated after the procedure.

At post-mortem examination the thymus and parathyroid glands could not be identified grossly or in serial sections of the mediastinal tissue, neck organs and pharynx. There were no other congenital anomalies. Lymph nodes were enlarged; the deep cortical zones were depleted of lymphoid cells, and lymphoid follicles of the outer cortex were small and devoid of germinal centers. Only rare cells stained for IgG or IgM. The lymph nodes showed the same predominance of macrophages and eosinophils as the biopsied nodes. In addition, lymph nodes from all areas contained large multinucleated giant cells without cytoplasmic or nuclear inclusions or associated necrosis. The follicles of the spleen were poorly developed, with hypocellular periarteriolar zones and depleted peripheral zones. There were no germinal centers. The lymphoid tissue of the intestine was barely discernible and cytologically inactive.

Table 2. PHA-Stimulated Lymphocyte Transformation of Peripheral Blood Lymphocytes after Fetal-Thymus Grafting.

Source	Time after Grafting (%)								
	0 hr	6 hr	12 hr	18 hr	24 hr	36 hr	48 hr	3 days	5 days
Patient cells with PHA	4*	38	35	42	42	40	54	48	52
Patient cells without PHA	3	2	2	3	2	2	4	2	2
Control cells with PHA	65-87								
Control cells without PHA	1-5								

*% transformed lymphocytes/100 mononuclear cells examined.

The lungs contained a patchy infiltrate composed mainly of macrophages. In addition, multinucleated giant cells were noted in some alveoli and bronchi. Similar cells were seen in the heart, trachea, esophagus and skin. Light and electron microscopy revealed no viral-type inclusions, acid-fast bacilli, fungi, or bacteria. Viral cultures of lymph nodes and spleen were negative.

The Millipore chamber was intact, and the enclosed thymus apparently viable. The lobular architecture was intact and was consistent with that described for a gestational age of 12 to 13 weeks[16]; there was no differentiation into cortical and medullary zones, and the lobules consisted of lymphoid cells and rare epithelial cells. Hassall's corpuscles were not present.

Discussion

The thymus and parathyroid aplasia occurring in maternal half-siblings, confirmed at autopsy, represents an unusual example of these associated anomalies, which were originally described by DiGeorge in the syndrome bearing his name. Since these siblings were of opposite sex an autosomal dominant mode of inheritance is suggested; the rare sex-linked dominant pattern, however, cannot be excluded. Moreover, the finding of a partial deficiency in thymic and parathyroid function in the mother, in conjunction with nonidentical paternity, strongly supports maternal transmission of the defect.

At present, thymus implantation offers the best hope of survival for these patients.[17] The present availability of sterile fetal tissues makes this mode of therapy feasible. The greatest risk from such an implant is the initiation of a fatal graft-versus-host reaction. The use of fetal thymus for reconstitution is based on the concept that fetal thymic cells are incapable of initiating such a reaction. Fetal thymuses 13 and 16 weeks old were used in two previous successful implants, and no graft-versus-host reactions were observed[2-4]; immature fetal tissue, therefore, may offer some protection from this complication. Since the graft-versus-host reaction requires contact of donor lymphocytes with host cells, the use of thymus in a Millipore diffusion chamber in the

present report obviated the risk of this reaction. Moreover, this technic permits the use of more mature thymic tissues in reconstitution.

Experimental studies with thymectomized animals have demonstrated at least partial improvement of cell-mediated immune function after implantation of mature thymus in diffusion chambers,[5-7] suggesting that the elaboration of a humoral factor is an important function of the thymus. Our data support those in the laboratory animal. In our patient, rapid acquisition of PHA responsiveness of the lymphocytes as early as six hours after implantation is best explained by the action of a potent humoral factor. No blood products were given to account for the rapid conversion. Since this child died nine days after implantation, it is not possible to ascertain the extent of immunologic reconstitution. However, it is clear that the use of fetal thymus of 13 to 16 weeks, even in a diffusion chamber, had a considerable influence on the lymphocytes responsible for PHA responsiveness.

The existence of a thymic hormone is a controversial issue. In the mouse, it is believed that the cell of origin may be the epithelial elements of the gland. Examination of the thymus three or more weeks after implantation in Millipore chambers revealed a predominance of epithelial elements. In contrast in the present study, small lymphocytes predominated.

The nature of the immunologic defect in these infants poses yet another problem. Proved congenital absence of the thymus and parathyroid glands in both children justifies their inclusion in the syndrome described by DiGeorge. Adequate humoral immunity is suggested by the normal appearance of immunoglobulins with maturation, a vigorous response to typhoid immunization, and normal numbers of circulating lymphocytes bearing membrane-associated immunoglobulins. It remains unclear, however, whether these half-siblings also had a defect of humoral immunity and therefore could represent a combined immunodeficiency syndrome. Histologic examination of lymph nodes, spleen and intestinal lymphoid tissue suggests a quantitative deficiency of thymic-independent (B) lymphocytes as well. However, the discrepancy between the ability to synthesize gamma globulin and lymph-node morphology in response to immunization may be explained by the lag period known to occur between these two features.[5] An interval between functional reconstitution and its anatomic reflection in lymphoid tissues after thymus implantation has also been described in the DiGeorge syndrome attributed to the absence of T lymphocytes.[18] Interactions between antibody-producing (B cells) and thymus-dependent lymphocytes (T cells) appear to be important in the elaboration of both humoral and cell-mediated function.[19] The extent of thymic regulation for the development of antibody function appears to vary with the species of animal, and the antigen employed; the age at the time of testing may also be important.[20]

Histologic descriptions of the lymphoid organs in previous reports of DiGeorge's syndrome emphasize normal populations of lymphocytes and plasma cells in the thymic-independent regions.[2-4,21] Such patients were older than those described above, and thus the morphologic findings may not be comparable. Moreover, many of the previous cases may have represented thymic hypoplasia rather than aplasia. In a previous report of thymic hypoplasia associated with thyroid hypoplasia, an underdevelopment of the thymic-independent regions of lymph nodes was also noted.[22]

An abundance of eosinophils and macrophages was noted in many tissues of both siblings. Eosinophilia has been described in patients with cell-mediated, humoral or combined immune-deficiency states and is frequently associated with *Pneumocystis carinii* infection.[23] The sibling of our patient illustrated this finding. Macrophages as well as eosinophils are known to possess phagocytic properties. Macrophages appear to be important in the inductive phases of the immune response and in antigen processing. Since antigens associated with macrophage membranes are those that more commonly induce cell-mediated immunity,[24] it is not surprising that an increase in these cells may be observed in patients with cell-mediated immune deficiency. Alternatively, this increase in macrophages and eosinophils might also represent a compensatory replacement for nonfunctional cell types.

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TOXIGENIC *ESCHERICHIA COLI*

A Cause of Infantile Diarrhea in Chicago

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Abstract Approximately 1000 children per year are admitted to Cook County Hospital with acute diarrhea. The etiologic agent cannot be found in the majority of cases. Six hundred strains of *Escherichia coli* were isolated from the stools of 29 children with diarrhea not yielding salmonella and shigella. Classic serotypes of enteropathogenic *Esch. coli* (EPEC) were found in nine of 29 cases. *Esch. coli* enterotoxin was assayed in an infant rabbit model; strains causing fluid accumulation and distention of the small bowel were found in 24 of 29 cases. This reaction could be induced by sterile filtrates as well as viable cultures. Two or three enterotoxin-positive strains were often present in the same stool specimen. Simple serotyping for EPEC identified pathogens in 31 per cent of cases of diarrhea whereas the animal model incriminated pathogenic strains in 83 per cent of cases.

DESPITE advances in nutrition and sanitation, infantile diarrhea remains a major problem in the United States. At the Cook County Pediatric Hospital, we admit approximately 1000 children each year for dehydrating diarrhea. Several thousand other children are treated in the outpatient clinic. Gordon has pointed out that the mortality from acute diarrhea has progressively declined in the United States over the past 70 years.[1] There has also been a decreased incidence and a change in the seasonal prevalence: formerly termed "summer diarrhea," the disease now has its highest incidence in the winter.

Many micro-organisms have been associated with acute diarrhea. Among the bacteria these include salmonella, shigella, enteropathogenic *Escherichia coli* (EPEC) and vibrios. Protozoa such as ameba and giardia and enteroviruses can also cause the acute symptoms. With this imposing list, it is surprising that a specific pathogen cannot be identified in as many as 80 per cent of cases of acute diarrhea.[2-9] The laboratory usually signs out the stool specimen as "normal flora."

Studies from India[10-12] and Vietnam[13] have shown that enterotoxin-producing strains of *Esch. coli* can cause acute diarrhea in adults. The somatic serotypes of these strains have not generally been the recognized EPEC. The toxigenic strains often masquerade as benign constituents of the normal flora.

At present, *Esch. coli* enterotoxin can only be assayed in an animal model. Although this test is cumbersome, the results suggest that enterotoxin production is more important in determining pathogenicity than the somatic serotype or in vitro biochemical reactions.

We are reporting our search for enterotoxin-producing *Esch. coli* in the fecal specimens of children admitted to Cook County Hospital with acute diarrhea. These strains appear to be an important cause of infantile diarrhea in Chicago.

METHODS AND MATERIALS

Thirty-three children, one month to four years of age were initially studied. All were admitted to Cook County Pediatric Hospital for dehydration and toxicity due to diarrhea. Excluded from the study were children with chronic symptoms (over five days) or with a history of antibiotic treatment. Stools were collected on admission and during the second to the fourth week of convalescence.

Seventeen children were studied in July, 1970, and 16 in January, 1971. Preliminary bacteriologic analysis revealed two cases of shigella and two cases of salmonella; the remaining 29 cases are the subject of this report.

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