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ABSTRACTS

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Plenary Sessions

17 *Measles Antibody Level as an Aid to the Diagnosis of Subacute Sclerosing Panencephalitis.* Clarence J. Gibbs, Jr.*, D. Carleton Gajdusek, John Hooks*, Armand Lowenthal* and Barry R. Adels*, National Institutes of Health, Bethesda, Md., Institute Bunge, Antwerp, Belgium and Children's Hospital, Boston, Mass.

Subacute sclerosing panencephalitis (SSPE) is a clinically and pathologically distinct disease of children and adolescents caused by measles virus, as recently proved by viral isolation in several laboratories from brain biopsies. It might now better be called 'delayed (and slow) measles encephalitis'. Specific CF, HI, FA and neutralization antibody levels to measles are usually in a hyperimmune range far above the mean titers of late convalescent measles sera tested in our laboratory. In 54 of 72 patients, serum CF and HI antibodies to measles were in this hyperimmune range. CSF had significant measles antibody titers in 47 of 51 patients, although titers were lower than simultaneous serum titers. In a 'blind' control study, measles CF and HI antibody were measured in 136 sera from 98 patients and 180 CSF from 162 patients of all age groups with a wide variety of CNS diseases. There were 65 serum-CSF pairs from the same patients. In the coded series, 49 sera were from 32 patients and 29 CSF's were from 20 patients with SSPE. Of the 180 CSF specimens, the only ones with detectable measles CF antibody were 25 of the 29 specimens from 18 of the 20 SSPE patients. Measles antibody titers[1] in CSF ranged from 2 to ≥ 40 by CF or HI or both. Of the 136 sera studied, 33 of the 49 specimens from 19 of the 32 SSPE patients had measles CF antibody titer of 160 or higher; six had titers of 40 or 80, three of 10 or 20, and only one was negative at 10 (6 were anticomplimentary). In contrast, of the 87 sera from non SSPE patients, only two had titers of ≥ 80, six had titers of 40–80, 22 had titers of 10 to 20 and 50 were negative at a 10 dilution (seven were anticomplimentary). In the two instances where serum titers were ≥ 80, one was from a patient late in acute measles and the second was from a child with Guillian-Barré syndrome. Serum titers of ≥ 1:160 were found only in SSPE patients. Measles antibody estimation on serum and CSF can thus identify most suspected SSPE cases and confirm diagnosis arrived at from clinical and other laboratory data, obviating the need and risks of brain biopsy. In spite of autopsy confirmation of SSPE, a significant number of brain biopsy specimens from patients with this disease fail to reveal intranuclear (type A) inclusions and are reported as chronic encephalitis of unknown etiology.

* By invitation

[1] Titers are expressed as reciprocal dilutions.

Discussion

Vincent A. Fulginiti (University of Arizona, Tucson): The only other place in measles that I have seen titers of this magnitude has been in the child with atypical measles following killed measles virus vaccine, and then challenged with natural disease, and here the titers both of neutralizing and HI antibody are phenomenal, as high as 1:128,000.

Would you comment on what you might see as any similarity in the pathogenesis of these two conditions.

D. Carleton Gajdusek: High antibody titers similar to those of SSPE have been seen only in patients challenged with natural measles after immunization with killed measles vaccine. Some patients with SSPE developed neutralization and fluorescent antibody titer to measles of well over 1:16,000, and HI titers are correspondingly high.

Samuel L. Katz (Duke University School of Medicine): Among your controls, you included patients with multiple sclerosis and found no measles antibody in their CSF, yet we have heard from other laboratories reports of measles-specific antibody detected in the spinal fluid of some patients with multiple sclerosis.

Could you comment on this discrepancy?

Dr. Gajdusek: In multiple sclerosis we have the only other disease in which a high measles virus antibody has been reported to occur in the cerebrospinal fluid. This has usually been a positive reaction only in undiluted spinal fluid, occasionally at a 1:2 to 1:10 dilution. Higher titers at the sensitivity of our antibody assay system are unusual in multiple sclerosis. In SSPE the spinal fluids were usually positive at 1:10 dilution or greater. We are dealing with another order of magnitude of antibody level from that usually seen in multiple sclerosis.

Also, elevations of serum measles antibody in multiple sclerosis patients were only about 10% higher than those in the normal control series, whereas in SSPE, we are encountering a geometric mean measles CF or HI titer 4- to 16-fold or more above that found in normal control subjects.

Jack Singer (National Institutes of Health): With the large number of children receiving the attenuated rubeola vaccine at an early age, and the long latent period prior to the onset of neurological symptoms of SSPE, would you expect an increased incidence of SSPE?

Dr. GAJDUSEK: The vaccine and SSPE incidence: Anywhere in the United States that we hunt for patients, we find a rapidly increasing rate of SSPE. I suspect that this reflects pediatric and neurological awareness of this diagnosis. As a rough and yet unverified estimate of the incidence, we expect to find an active case for every million general population served by a pediatric neurological center. We estimate that about 200 cases have died *per annum* in recent years in the United States, in spite of much lower earlier estimates of the mortality from this disease.

I expect that we will find increasing incidence everywhere the disease is sought for, and I believe it should be reportable and recorded very seriously from now on. The possibility of its developing from the vaccine virus certainly cannot be denied at the present time. The population has been extensively vaccinated, yet natural measles is still endemic. We know of cases that have followed natural measles by a few months; more commonly, it occurs several years after natural measles. A case has been reported in the young child of a physician who had not had antecedent clearly recognizable measles, but who was serologically immune. Several cases have now occurred a few months to over a year after the use of live attenuated measles vaccine without knowledge of whether there was antecedent unrecognized measles infection or subsequent suprainfection with natural measles virus.

If extensive use of vaccine markedly reduces or eliminates measles virus circulating in the country, the incidence of SSPE should drop off after a lag period of a few years. If its incidence fails to fall once measles is nearly or completely eliminated, obviously we then would suspect the vaccine virus.

Epidemiological surveillance of SSPE incidence is a very urgent matter; at the present time, our knowledge of its true incidence is very uncertain.

HAROLD LISCHNER (St. Christopher's Hospital, Philadelphia): I wonder if you have noted any familial incidence of SSPE or any association with chronic mucocutaneous moniliasis. We have seen one family with chronic mucocutaneous moniliasis in which one child developed SSPE. We are interested in the cellular immunity to measles in these children. Have you used techniques similar to those reported by Dr. SAUNDERS?

We find that in one of two children with SSPE studied in multiple tests on three separate occasions, we were unable to demonstrate any responses in lymphocyte cultures *in vitro* to measles antigens. This child was from a family with mucocutaneous moniliasis. It is conceivable that the pathogenesis was different, but the fact that there does seem to be specific unresponsiveness of the lymphocytes of this child to measles antigen, I think, makes it imperative that this kind of study be done on other patients with the same disease.

It is conceivable that in our one child who did respond, as well as in Dr. SAUNDERS' child, the lymphocytes were responding to other tissue antigens in our measles preparation, and that, actually, there is specific cellular unresponsiveness in the patients with SSPE.

Dr. GAJDUSEK: No, we have had no familial cases yet, and the association of SSPE with chronic moniliasis I was unaware of.

There has been much speculation about the possibility that cellular immunity specifically to measles is impaired in SSPE patients. The hypothesis has been repeatedly made that the disease occurs, perhaps, in those individuals who either lose or lack quantitatively a sufficiently strong delayed hypersensitivity immune response to measles. How this is to be tested is a great problem. There is no adequate measles skin testing antigen available at present which has been sufficiently controlled. We are reluctant to use such antigens on previously vaccinated children, particularly those immunized with killed virus vaccine. The mixed reports of responsiveness and lack of responsiveness of patients to measles antigens must be taken with caution.

The SSPE patients with a hyperimmune serum antibody response might have a local Arthus phenomenon to the skin testing antigen. A biopsy of the skin of any positive test should surely be taken to evaluate it histologically. Using leukocytes *in vitro* to test their sensitization to measles antigens or to test their ability to attack a sheet of HeLa cells containing incomplete measles virus, in which measles antigen and defective virus are synthesized without the shedding of infectious virus, as BELLONTI and RUSTIGIAN have proposed, have thus far failed to give a conclusive answer to the problem of whether there is a specific defect in cellular immunity to measles in SSPE patients. False positive and false negative reactions may easily occur with these techniques, particularly in view of the high levels of circulating antibody. What percentage of the normal population may show a defect of delayed hypersensitivity to measles after natural measles infection we do not know yet.

PEARAY L. OGRA (State University of New York, Buffalo): In view of these disproportionately high antibody titers in the spinal fluid, one would imply that at least part of the spinal fluid antibody is produced locally in the central nervous system.

Did you study the total immunoglobulin content in the spinal fluid in these patients, and what was its proportion to the serum immunoglobulins? Further, do you have any data on the immunohistologic localization of specific measles virus antibody and the tissue localization of immunoglobulins in the tissues of the central nervous system?

Dr. GAJDUSEK: We are not working specifically on that problem in our laboratory. On the other hand, there has been work in Dr. KOLAR's group, and in hospitals in Europe, indicating that measles antibody is being produced within the central nervous system and that the high levels in the CSF are not entirely from a passage of the serum antibody into the spinal fluid.

It was with this in mind that some patients were treated with x-irradiation of the head in an attempt to surpress antibody formation within the skull. I do not believe it was of much help.

Measles antigen has been localized by immune fluorescence in the inclusions and intracytoplasmically; in fact, it was this detection of specific measles antigen in the brain by immunofluorescence, together with the demonstration of a hyperimmune response to measles in the serum, which led to the first incrimination of the measles virus in SSPE. The virus isolations have now proved that the virus is typical measles.

Finally, TER MEULEN and RUCKEL-ENDERS in Göttingen have shown the presence of large amounts of measles antigen in neuronal cytoplasm; whether it is complete or incomplete defective virus has not yet been established. Immune fluorescence to detect immunoglobulins and the third component of C′ (com-

plement) gives the same extensive floccular staining of antigen-antibody complex in the neurons. Removal of immune globulin by buffer of low ionic strength and low pH permits the demonstration in the same location of specific measles antigen.

18 *Deficiency of Chemotactic Function in the Human Neonate. A Previously Unrecognized Defect of the Inflammatory Response.* MICHAEL E. MILLER*, Children's Hospital of Philadelphia, Pa. (introduced by William J. Mellman).

In recent years, considerable attention has been focused on the relatively late inflammatory events of phagocytosis and intracellular killing of ingested organisms. In mobile cells, chemotaxis is an important prerequisite for phagocytosis, for cells must approach the site of infection before engulfment of organisms can begin. In these studies, chemotactic function has been compared between neonates, a group with known increased susceptibility to bacterial infections, and adults. An *in vitro* modification of the Boyden assay was developed which made possible the use of *human* leukocytes (PMNS) and serum throughout. The *humoral* and *cellular* components of the chemotactic response were *individually* considered. The data showed that: 1. When incubated in the presence of chemotactic factor generated from standard pooled sera, neonatal PMNS showed significantly less chemotactic migration than adult PMNS. This was true whether the serum factor was generated from gram+ or gram− bacteria or antigen-antibody complexes. 2. When incubated in the presence of a standard PMN suspension, neonatal serum was a much poorer source of chemotactic factor (CF) than equal amounts of adult sera. This was true for bacterial or antigen-antibody generating agents. The addition of purified C'3 to neonatal serum prior to generation did not increase the yield of CF. These data demonstrate two basic deficiencies of the neonatal inflammatory response and their application may provide valuable insight into the management of neonatal infections.

Discussion

WILLIAM T. KNIKER (University of Arkansas Medical Center): Have you tested the sera of newborns for total hemolytic complement activity, since immune hemolysis requires the participation of all nine components? If the hemolytic activity is depressed, that would be further evidence for deficiency in one or more of the later components beyond C'3.

It is always appealing to consider that a newborn might be deficient in some component or enzyme. Yet, the possibility exists that an inhibitor or inactivator of newborn or maternal origin might be present in the serum and associated with the polymorphs. Have you ruled out the presence of an inhibitor?

MICHAEL E. MILLER: With reference to the first question, total hemolytic activity, as studied both in our laboratory and others, does reveal approximately a 60 to 70 % level of that seen in the normal adult in newborn sera. We have not yet done quantitative studies, to look at the relative quantitative amount of C5 involved in the newborn in hemolytic activity.

With regard to the second question, while our data are not complete, we would suggest that there is not an inhibitor present. If one takes normal adult sera and dilutes it out in two separate ways, first by adding just a normal saline diluent, and secondly by putting progressively increasing amounts of newborn sera into it, one will find that the titer, or activity, in both of these systems drops off considerably more rapidly when a simple diluent is added.

In other words, adding more and more newborn sera does not cause an accelerated decrease in these results, as we might expect with an inhibitor.

DAVID G. NATHAN (Children's Hospital Medical Center, Boston, Massachusetts): I have a source of confusion, myself, about studies comparing newborn cells with normal cells; and that, of course, is the difference in distribution of types of cells in the leukocyte mixture.

Did you start in the newborn with a different concentration of polys in the mixture, such that one might expect fewer to come through the millipore, no matter what the control situation might be?

This has just been a technical problem for those who deal with this particular area, and I wonder if you can comment on it.

Dr. MILLER: Yes. This is a very highly standardized system in which the concentration of polymorphs is the standardized factor. In all preparations, a concentration of polymorphs is first prepared at 5 million polymorphs/ml.

We have also looked intensively at the effects of other cell members in the population, including relatively young polys, eosinophils, lymphocytes, red cells. With both of these systems, the results are entirely independent of the presence of these other cell types within the polymorph suspensions.

CHESTER ALPER (Children's Hospital, Medical Center, Boston, Massachusetts): Have you determined C5 levels either immunochemically or functionally in neonatal serum? Secondly, have you tried to correct the deficiency of chemotactic function in neonatal serum by the addition of C5-deficient mouse serum?

Dr. MILLER: We have not yet measured C5 levels in neonatal serum.

In this particular system, with the newborns, we have not tried the mouse deficient serum, but in the patient with the C5-deficient function, mouse sera from a C5-deficient strain, such as A/Tax, does not correct the defect, whereas normal complement containing mouse sera will do so.

FREDERIC M. KENNY (Children's Hospital, Pittsburgh, Pennsylvania): Did you detect any difference between the leukocytes of male versus female infants?

Dr. MILLER: There were no differences here, nor were there any other differences seen in the group, in reference to birth weight, age, and so forth.

DAVID W. SMITH (University of Washington Medical School): Have you studied babies at, say, 7 days of age or 14 days of age to see if this is a phenomena limited to the immediate neonatal period? Or is it a developmental phenomenon, gradually improving with time?

Dr. MILLER: We have done too few even to speculate on it.

FREDERICK C. BATTAGLIA (University of Colorado Medical Center): How does one correct for differences in cell size? I mean, how dependent is this measurement on the pore size you choose for the screen—in this case, 3 μ?

Dr. MILLER: The pore size of the filter does not appear to be a factor in these results. The background level, or, that number of polymorphs that migrate through the filter in the absence of chemotactic factor in the lower chamber, is the same for newborn and adult cells.

RICHARD L. DAY (New Rochelle, New York): Did you test any babies who had had an intrauterine infection?

Dr. MILLER: Only one, and unfortunately we did not have leukocytes from that baby.

19 *'Silent' CNS Disease of Neonates: A Prospective Study of Infants Born with Increased Levels of IgM.* CHARLES A. ALFORD, JOHN W. FOFT*, SARAH S. POLT*, GEORGE CASSADY*, Departments of Pediatrics and Clinical Pathology, University of Alabama Medical Center, Birmingham, Ala.

2916 cord sera obtained from all deliveries in 1 year were screened for elevated levels of IgM. The study population was derived primarily from a low income, Negro group. Elevations which persisted at least 4 days were detected in cord sera from 123 (4.2 %) of the newborns. To define infection, these infants and a control group of 386 consecutive deliveries were examined serially from birth up to 18 months by clinical, immunologic, serologic and microbiologic means, regardless of clinical status. Infections, mostly subclinical forms, were proven in 42 (34 %) of the 123 newborns with ↑ IgM (cytomegaloviral 8, urinary tract 8, toxoplasma 6, rubella, enteroviral, and syphilitic 2 each; miscellaneous 14). In contrast, comparable infections were demonstrated in only 3 (0.8 %) of 363 control infants with normal IgM. Thus, rate of proven infection was 42-fold higher in newborns with ↑ IgM.

Although symptoms were absent, CSF pleocytosis and ↑ protein values persisting up to 5 months were detected in 10 of 20 newborns with systemic congenital infection (toxoplasma 6/6, cytomegaloviral 2/8, enteroviral and syphilitic 1/2 each) and in 4 other infants with ↑ IgM. Two of these with toxoplasma and 2 with cytomegaloviral infections have already developed signs of CNS damage during the first year. Data indicate that subclinical congenital infections with 'silent' CNS involvement occur frequently in certain newborn groups and may be an important cause of brain damage that is currently unclassified. When coupled with methods for specific identification, screening of cord and neonatal sera for ↑ IgM can be helpful in the search for and definition of this type of disease.

Discussion

ALEX J. STEIGMAN (Mount Sinai School of Medicine): In view of its possible bearing on the site of production, have you been able to determine the relative quantitative proportion of the various immunoglobulins in the spinal fluids studied?

CHARLES A. ALFORD: No attempt has been made to determine the nature of globulins in the spinal fluids collected from infants presented in this study. However, we have examined them in a case of neonatal herpes simplex infection with severe central nervous system involvement and high levels of total spinal fluid protein. In this instance, though quantitatively less, the qualitative pattern of IgM, IgA, and IgG, as determined by immunoelectrophoresis of concentrated spinal fluid, was similar to that detected in the sera simultaneously collected; the changes that occurred were also parallel to the serum changes.

E. RICHARD STIEHM (University of Wisconsin Medical School): There seems to be a marked difference in the incidence of elevated gamma M from population to population. Dr. NICHOL in my laboratory recently completed a survey very similar to your own, studying over 1000 consecutive newborns, and her incidence of elevated macroglobulins was only at the 1 % level. So I think that your delineation that this is a low income group is very pertinent.

Dr. ALFORD: During the first year of these studies, as noted here, cord IgM elevations were detected in 4.2 % of the population; in the second 9-month interval, they were demonstrated in only 3 %. Thus, it appears that the number of neonates born with increased levels of IgM may vary from year to year even in the same population. Perhaps it is not surprising, then, that great variations may be found in population groups from different locales and from different socioeconomic backgrounds. Infections may not be the only factor that increase IgM production *in utero*. Therefore, I would venture to guess that even the percentage of infections detectable in neonates with increased levels of IgM may differ from one newborn group to another. Because of the concentrating effect, cord IgM screening may assist in determining the frequency of subclinical congenital infection in any given population providing specific efforts are made to uncover the infections.

WILLIAM OH (Michael Reese Hospital): Have you tried to correlate the incidence of infection with the birth weight, with particular reference to low birth weight infants.

Dr. ALFORD: There appears to be a slight, but probably significant, increase in low birth weight infants among the total group born with elevated levels of IgM. This was observed mainly in those in whom no specific diagnosis has yet been established. In neonates with subclinical congenital infections and elevated levels of IgM, the incidence of low birth weight infants was not increased. The limited data in this study does, however, suggest that if infected infants are prematurely born or 'small for dates', they may have a greater tendency to develop signs of disease early in life, probably because of a greater degree of tissue damage.

JUNE P. BRADY (San Francisco General Hospital): I think this is a very important and very interesting paper, but if I understand you correctly, you did suprapubic taps and spinal taps on a group of normal infants, and I wonder what reason you gave to the parents for doing these studies.

Dr. ALFORD: Permission was gained to perform these studies. I feel very strongly that congenital infections in newborns must be diagnosed so that appropriate follow-up studies can be obtained for proper mangagement. Even if the infants appear normal, serial and careful audiometric, psychometric, and ophthalmologic examinations need to be performed so that the necessary educational programs can be planned for each of them in coming years. In some cases, such as those with subclinical toxoplasmosis and abnormal spinal fluid findings, therapeutic measures should be tried; they have not been adequately evaluated to date in this type disease because of lack of practical diagnostic methods.

The control spinal fluid and suprapubic urine data were obtained primarily from newborns who were originally examined because of maternal history (prolonged ruptured membranes or signs of acute infection) that suggested a potential infection in the newborn that could not be documented by laboratory or clinical means. In each of these cases, the laboratory work was identical to that performed in neonates born with increased IgM. It is routine in our services to attempt to exclude infection in any situation that suggests their possible occurrence.

VINCENT RICCARDI (Massachusetts General Hospital): Were any of the affected children related to each other, or did they have any affected relations elsewhere in the family?

Dr. ALFORD: Not that we could find.

JOHN STARR (Cleveland Metropolitan Hospital): You mentioned that among the infected infants, some already are showing signs of neurologic damage. Did you find any incidence of neurologic damage among the controls?

Dr. ALFORD: Yes, but the incidences in infected groups is much higher. However, I hasten to add that the number of infected infants detected to date is really too small to determine true significant differences. In addition, I find it exceedingly difficult to select a true control group for this type of silent disease. Since most of our infected infants were of normal size and appearance, theoretically, 26 consecutively delivered infants with normal levels of IgM could serve as a control. No evidence of neurologic damage occurred in the first 26 or even in the first 50 of our control group. In addition, tests currently available to assess minimal brain damage are very crude and need to be updated.

Clearly, the spinal fluid abnormalities detected here indicate that active CNS disease is present at birth in many of these neonates. However, the meaning of these findings with respect to CNS function in older infants and children is yet to be determined.

WILLIAM J. OLIVER (University of Michigan Medical Center): Would you comment on the portal of entry of organisms for those infants apparently born with a urinary tract infection?

Dr. ALFORD: In most of these cases, there was a maternal history of prolonged rupture of membranes (> 24 h) or signs of active infection in the mother soon after delivery. Such findings suggest that the infections are acquired *in utero* by the transplacental or ascending route. We have entertained the idea that they represent localization from previous fetal bacteremia, but this cannot be proven from our present data.

A urinary tract anomaly was detected in one of the eight infants with genitourinary infections, and another has had recurrent bouts of urinary tract infection in the first year of follow-up despite the fact that no congenital defect has been demonstrated on three separate occasions. The other six have remained normal after the initial infections cleared.

DOUGLAS SANDBERG (University of Miami School of Medicine): Are there differences in IgM serum concentrations from blood expressed from the umbilical cord when compared with blood drawn directly from the umbilical vein?

Dr. ALFORD: If possible, umbilical venous blood is obtained by aspiration. In difficult situations, it was collected by stripping the umbilical cord. In either event, contamination of cord by maternal blood should be excluded to avoid false positive elevations. These were detected in 6 % of our cord sera. When contamination occurred, it could be excluded by repeat sampling of neonatal sera for IgM and IgA within 24 to 36 hours in virtually all cases in this study. Thus, the contamination appeared to be due to collection errors or some unknown process of delivery that allowed for admixture of maternal blood rather than to intrinsic placental 'leaks' that occurred during the course of pregnancy as has been proposed by others.

20 *Immunobiologic Parameters Unique for the Placento-fetal Unit.* W. PAGE FAULK*, A-C WANG*, JOSEPH GOODMAN*, and H. HUGH FUDENBERG*, University of California Medical Center, San Francisco, Calif. (introduced by Melvin M. Grumbach).

This report deals with the normal biologic phenomenon of gestation. Twenty-five human placentae have been studied for immunoproteins with fluorescein labeled anti-human IgG, IgM, IgA, fibrinogen, and complement. Depositions of IgG, fibrinogen, and complement were found on the syncytial trophoblastic basement membrane and in areas of fibrinoid necrosis. This represents specific biochemical reactions, for they were eluted only with rigid immunochemical procedures. The eluates were studied in chromatographic systems, and fluorescein labeled molecules were found to return to the same anatomic areas in homozoic placentae. The proteins were detected as early as the first trimester and were not exaggerated in pathologic states.

Lymphocytes are immunocompetent cells and are sparsely found in normal placentae. Radioautographs were prepared with dextran separated fetal-cord lymphocytes from 10 human neonates using tritiated thymidine. The data revealed a unique fetal high labeling cell. Light and electron microscopic studies proved the cell to be hitherto unclassified.

The molecular substructure of fetal IgG was studied due to immunogenetic irregularities in the eluted placental IgG and the unusual morphology of fetal lymphocytes. Gel electrophoretic patterns of fetal gamma H-chains from 200 infants were compared to similar adult samples. An independent gamma fetal H-chain was discovered, but no L-chain differences were found. The data are presented as unique and hitherto undescribed cellular and biochemical phenomena of the normal placento-fetal unit.

Discussion

CHESTER W. FINK (University of Texas Southwestern Medical School): Since Type 0 mothers have been shown to produce isoagglutinins that have a high proportion of 7S antibody and Type A and B mothers make mostly 19S isoagglutinins, I wonder if there was any difference in the type of antibody found in the placentas of Type 0 versus Type A or B mothers?

W. PAGE FAULK: We were not able to find blood group isoagglutinins, and there were no immunoprotein variations.

JOHN B. ROBBINS (Albert Einstein College of Medicine): Have you demonstrated that fluorescein conjugates to serum proteins other than complement, fibrinogen, and gamma globulin do not stain these areas? And, is the eluted gamma globulin that you prepare from your placental extract binding to the basement membranes by virtue of its FAB fragment or in a nonspecific fashion by attachment to its H chain?

Dr. FAULK: All of these reactions were controlled by proper blocking and absorbing-out experiments. The reasons for the rigid controls were that we found that syncytial trophoblasts have a tendency to take up almost anything that has fluorescein on it, and we later were fortunate enough to find that this is a function of the fluorescein-protein ratio.

If we had a fluorescein-protein ratio greater than 3 we could stain the syncytial trophoblasts nonspecifically. We very gently conjugated our antisera with fluorescein so as to accomplish a fluorescein-protein ratio of less than 3. This was done according to the dialysis method of CLARK and SHEPARD [Virology *20:* 642, 1963].

We also looked at many other proteins. These included ceruloplasmins, transferrins, haptoglobins, etc. None of these tended to stick to the basement membrane when they were used with a low fluorescein-protein ratio.

We don't know if a 5S molecule, one which lacks an FC fragment, would mediate this reaction. I suspect it probably wouldn't, but I don't know.

J. BIENENSTOCK (McMaster University, Hamilton, Ontario, Canada): Could you re-present some of your evidence that these are true antigen-antibody reactions at the placental basement membrane? Could it not be a demonstration of placental basement membrane cell activity in transport of gamma globulin across the placenta? Have you looked for gamma A since this molecule hardly appears in cord serum and probably is not transported in a similar fashion to gamma G? If one looks at the adult gastrointestinal tract, for example, with an anti-gamma A anti-serum one can detect specific fluorescence along the basement membrane. We have taken this as evidence that there is some piling up of this similar globulin at the basement membrane before this molecule crosses the membrane into the intercellular spaces or epithelial cells on its way to the lumen.

Dr. FAULK: Do you extensively wash your gut basement membrane, and can you purify it, label it, and follow it back to homologous gut basement membrane?

Dr. BIENENSTOCK: The experiments referred to were performed with fluorescein-labeled antisera on adult tissue specimens. We have not done the elution and perfusion experiments that you have described.

Dr. FAULK: We have used IgA, IgM, IgD, and IgE. We find that IgG gives the best reaction. This IgG reaction can be accentuated if the eluate is put on a basement membrane that has been first washed at pH4 and then gradually brought back to pH 7.

I think that this is probably a specific immunochemical reaction. It can be blocked and absorbed out.

Dr. BIENENSTOCK: The specificity of the reactions that you describe is unquestioned. What I am querying is the certainty with which you regard this as an antigen-antibody reaction on the basis of your experimental results. Could it not be that you are simply demonstrating selective binding to receptors and transport of gamma globulin across the basement membrane of the placenta?

If one accepts that there is antibody to placental basement membrane, have you looked at the gamma globulin eluted from the placenta to see whether it reacts with lung and kidney, which also contain cross reacting antigens with the placental basement membrane.

Dr. FAULK: We have used these tracers on lung and kidney sections and they tend to stain.

I am sympathetic with your doubts of this being an immunochemical reaction. However, we have applied rigid immunologic controls and have demonstrated specificity within the limits of these controls.

GORDON B. AVERY (Georgetown University): I'd like to highlight the difference between the serological type of immune reactions and the cellular immune reactions involved in transplantation immunity, because, whereas you have shown evidence of some serological type of reactions, your sections also show a striking freedom of anything in the trophoblast, which does seem to form an immune barrier between the fetus and maternal blood, and besides that, there is very little evidence of the lymphocyte infiltrative processes that we normally associate with tissue rejection.

So that, whereas the basement membrane may be a site of some antibody deposition, the converse is also true: There is a striking freedom of cellular immunity, such as one sees with transplantation reactions within species.

Dr. FAULK: These are separate and possibly interrelated phenomena.

I don't know if the high index of DNA replication for the fetal lymphocyte means that these cells are being immunologically stimulated to replicate and subsequently to produce immunoglobulins. I accept your fact that there certainly seem to be tenuous relations between circulating antibody and delayed hypersensitivity.

JAY BERNSTEIN (William Beaumont Hospital, Royal Oak, Michigan): To get back to the question of localizing immunoglobulin in your sections, you showed fluorescence within a large mass of fibrin that you called 'fibrinoid'. The material seemed in your slide to reside in the intervillous space. It might have been deposited on a chorionic villus, where fibrin does accumulate and is not in contact with the fetal circulation.

Do you regard the deposition of fibrin on the maternal side of the villus to be also an immunologic phenomenon? In other words, is the demonstration of immunoglobulin necessary evidence of an antigen-antibody reaction?

Dr. FAULK: This is found in the areas of fibrinoid necrosis. You say these are in the intervillous spaces. I don't really know where areas of fibrinoid necrosis come from. We find areas of fibrinoid necrosis within the stroma of the normal villus very often. When this is found, it also stains. I don't know if this represents an area of earlier immunologic insult or not.

RICHARD HONG (Variety Club Heart Hospital, University of Minnesota): In regard to your heavy-chain findings, a few years ago we reported that in patients with hypogammaglobulinemia, there were changes in the heterogeneity of the total population, indicating a restricted mobility and a much greater tendency toward monoclonality. Subsequent to that time, Dr. ROWE in England reported similar changes in normal infants studied in the latter portion of the first year of life, and he felt that this was probably due to an emerging population of gamma globulin molecules. Because of limited stimulation resulting from the age of the children, there was not sufficient inducement to acquire the broad range of heterogeneity. It would look like your findings at an even earlier stage would support this. Do you think that the changes that you have in the fetal heavy chains might be due to lack of stimulation?

If the gamma globulin proteins present are representatives of some sort of an immunologic battleground, there might be changes in low birth weight infants or infants who have a compromised placental circulation. Have you seen differences in the patterns of staining in cases of that sort?

Dr. FAULK: What we would like to think is that this represents genetic information to synthesize a heavy chain *in utero* much like a fetus being able to synthesize fetal hemoglobin, and that following delivery or at a point soon thereafter, this gene would be turned off.

If this is true, then we might find fetal heavy chains in cases of adult-acquired agammaglobulinemia, and we are now looking at about 10 of these. I think we will

find this, but if we don't, then maybe it signifies a normal maturation process, as you suggest. If we do, it would perhaps indicate that this is a gene that is only active in the fetus.

JOSEPH BELLANTI (Georgetown University Medical Center): I have a comment that concerns your statement that an independent fetal H chain was discovered.

Could this be related to the manner of preparation? As I understand it, you compared the results by electrophoretic separation of eluted material compared with material from either maternal or paternal purified gamma globulin. Could this be a methodologic problem due to conformational changes of the eluted material?

Dr. FAULK: I suppose it could be, but it only could be if the conformational change that ensued would be a change that would occur more easily in fetal heavy chains than in adult heavy chains, because the adult heavy chains were prepared in exactly the same way, at exactly the same time, and in exactly the same quantities.

They behaved immunoelectrophoretically like normal IgG heavy chains, but they did not migrate in that way, indicating that the amino acid sequence of these heavy chains is perhaps different.

MICHAEL M. FRANK (National Institute, Agency for International Development and National Institutes of Health): What proportion of the gamma G eluted from the placental basement membrane goes back on the basement membrane, as compared with, say, normal serum gamma G?

Dr. FAULK: I really don't know. It doesn't all go back.

Gm and InV data do not account for all of the immunoprotein. It behaves antigenically like IgG heavy chains, but it does not behave immunogenetically like IgG heavy chains.

21 *Enhancement of Phagocytosis by Complement Components and Two Co-Factors.* RICHARD B. JOHNSTON, Jr.*, FRED S. ROSEN, CHESTER A. ALPER*, and MARTIN R. KLEMPERER, Children's Hospital, Boston, Mass.

The role of serum factors in promoting the phagocytosis of pneumococci was assessed by a spectrophotometric assay which depends upon reduction of nitroblue tetrazolium (NBT) dye. The validity of the assay was confirmed by a simultaneous measurement of the phagocytic index and by the uptake of I^{125}-labeled pneumococci by a monolayer of phagocytes. Bacteria prepared with γG antibody (Ab) were not phagocytosed unless a small volume of fresh normal serum was added. Fresh sera specifically deficient in C'2 or C'3 did not promote phagocytosis, whereas sera specifically deficient in C'5 or C'6 did. When highly purified components were added to the sensitized pneumococci, enhancement of phagocytosis was achieved only by the sequential addition of C'1, C'4, C'2, and C'3. Furthermore, a heat-labile, dialyzable serum cofactor which has been shown to enhance peptidase activity enhanced the phagocytosis of pneumococci-Ab-C'1, 4, 2, 3. Glycyl-tyrosine and glycyl-leucyl-tyrosine, which are substrates for C'3 peptidase activity, inhibited phagocytosis. In order to achieve enhancement equivalent to that of normal serum, a heat-labile, 5-6S, β-pseudoglobulin, which is not a complement component, was required in addition to C'1, 4, 2, 3 and the dialyzable co-factor. This protein probably stabilizes bound active C'3, and appears to be that protein absent from the serum of a previously described patient with recurrent bacterial infections.

Discussion

ROGER E. SPITZER (University of Cincinnati College of Medicine): Do you have other evidence to support the fact that this is a C'3 stabilizing factor?

RICHARD B. JOHNSTON: Yes, we do. Most of this was obtained from experimental systems other than measurement of the enhancement of phagocytosis, however. It developed in the course of evaluating the young man with rapid catabolism of C3 and recurrent infection, whose complement-mediated functions were abnormal until this protein was added to the test system.

Let me say that, as it stands now, C3 'stabilization' is an hypothesis, and its exact proof is lacking.

Dr. SPITZER: It's a very intriguing prospect. Tomorrow, Dr. VALLOTA, in one of the specialty sessions, will present a paper on a factor found in nephritic sera that combines with a cofactor similar in many respects to the one you have just described. The major difference, however, is that this combination of nephritic factor and cofactor produces an enzymelike substance which lyses serum C3 rather than stabilizing it.

Dr. JOHNSTON: We would hypothesize that our pseudoglobulin, as I stated, does the opposite, perhaps in a fashion analogous to the effect of the C1 esterase inhibitor on C1 esterase, that is, the 5-6 S beta-pseudoglobulin may inhibit the enzymatic inactivation of C3.

However, there are similarities in the physico-chemical properties of these two pseudoglobulins and also the so-called C3 inactivator, the conglutinogen activating factor.

There are a lot of pseudoglobulins that are in orbit around C3 that are yet to be clearly defined.

Dr. SPITZER: But there aren't very many that participate in the formation of a C3 lytic enzyme as with our nephritic patients and possibly in the patient you describe.

Dr. JOHNSTON: The evidence that we have in regard to that patient—and this presentation was not about that patient—shows that, indeed, he does have a very rapid turnover of C3, but that infused normal plasma slows this inactivation dramatically.

JOHN ZUCKER (Mount Sinai Hospital, New York): Have you had a chance to study any patients with sickle cell disease, in view of recent reports of a defect in opsonization of pneumococci in patients with this disease?

Dr. JOHNSTON: Yes, we have, using this system. There are some patients whose sera perform abnormally. We haven't found this to be as frequent a defect as did WINKELSTEIN and DRACHMAN, nor can we relate the presence of an opsonization defect to an increased incidence of infection, in particular, pneumococcal infection. I don't know yet quite what that means.

JACK METCOFF (Michael Reese Hospital): Your interesting paper called to mind a lovely talk by Dr. KRETCHMER yesterday in which he showed a picture of six blind men looking at an elephant, and I wonder if you can help at least one blind man get his hand on the shoulder of another.

It has been demonstrated that phagocytosis is an energy requiring phenomenon. VILLEE and others have suggested that phagocytosis with energy (ATP) utilization and with the generation of ATP will be associated with glycolysis. You have demonstrated that complement—some specific component of complement

—is an important factor in phagocytosis. Is there a relation between glycolysis and energy generation or utilization and complement utilization or generation?

Dr. JOHNSTON: That's a fascinating question, and I don't know the answer, in short. There is a hint in the literature that complement does have some role in what goes on after the ingestate is ingested. We have not tried to separate the effect of complement on ingestion from any effect it might have on subsequent intraphagocytic events.

22 *Chronic Mucocutaneous Candidiasis: Definition of a Cellular Immune Deficit.* RICHARD A. CHILGREN*, HILAIRE T. MEUWISSEN*, PAUL G. QUIE, ROBERT A. GOOD and RICHARD HONG, Variety Club Heart Hospital, University of Minnesota College of Medical Sciences, Minneapolis, Minn.

Six cases of generalized mucocutaneous candidiasis associated with cutaneous anergy have been reported. Paradoxically, leukocytes from 5 of these 6 patients respond *in vitro* to stimulation with phytohemagglutinin (PHA) and/or *C. albicans* antigen with blast transformation and thymidine uptake. We have attempted to passively transfer delayed hypersensitivity to 2 of these patients by intracutaneous and subcutaneous injection of 2×10^8 lymphocytes from 2 normal donors:

Patient	Donor skin test		Patient skin test	Post transfer
	C. alb.	PPD	C. alb.	PPD
I	Pos.	Pos.	Pos. Day 1–14	Pos. Day 1–14
II	Neg.	Neg.	Pos. Day 11–14	Neg.

These results suggest that the presence of normal sensitized cells causes temporary reversal of the anergy, allowing cutaneous manifestation of delayed hypersensitivity. In patient II, donor cells appear to have become sensitized in the recipient after 10 days. Normal cutaneous delayed hypersensitivity seems to require interaction of antigen with sensitized lymphocytes causing release of a chemotactic factor, probably migration inhibitory factor (MIF), which leads to mononuclear cell infiltration. The *in vitro* studies suggest normal antigen-lymphocyte interaction in these patients, and their cutaneous response after donor cell administration implies normal mononuclear infiltration. Therefore, we postulate a defect in the chemotactic phase of cutaneous delayed hypersensitivity, i.e. either a deficient, defective or inhibited MIF or other chemotactic agent.

Discussion

HAROLD LISCHNER (St. Christopher's Hospital, Philadelphia, Pennsylvania): Dr. CHILGREN, we have talked about this, but I'm a little confused on a couple of things.

Number 1, the transfer factor you gave, that you mentioned at the last, produced positive skin tests in the patient who apparently did not make MIF. Do I have my patients right?

RICHARD A. CHILGREN: That is correct. There have been additional developments since submission of this abstract. First, we have added one patient with chronic mucocutaneous candidiasis, a 25-year-old male, who also has Addison's disease, giving us a total of three patients. Second, while patient II was originally anergic and temporarily became skin test positive following administration of viable lymphocytes, she was lost to follow-up after two negative skin tests. Now, 1 year later, she was converted to a positive *C. albicans* skin test. I cannot explain this, except to speculate that the original cellular administration served as a source of transfer factor.

In collaboration with Drs. Ross ROCKLIN and JOHN DAVID in Boston, we measured the Migration Inhibitory Factor (MIF) production in these patients. Drs. ROCKLIN and DAVID did the measurements in their laboratory on patients I and II. Cells from patient I failed to produce MIF in response to stimulation by *C. albicans* and streptokinase and streptodornase, while patient II, now skin test positive, did produce MIF. These tests were confirmed in our laboratory. We also tested patient III, and found that his lymphocytes did not produce MIF. Therefore, the MIF production correlates with the skin test results in these patients and suggests that some patients with cutaneous anergy and chronic mucocutaneous candidiasis have decreased or absent production of MIF.

With that background, I can directly answer your question concerning the transfer factor. Dr. JOHN DAVID prepared and administered transfer factor to patient I. LAWRENCE *et al.* demonstrated a number of years ago that delayed hypersensitivity could be transferred passively through administration of the dialysate of an extract of sensitized WBC's. While patient I was anergic and failed to produce MIF prior to administration of the factor, within 1 week after receiving it, skin tests to *C. albicans* and PPD showed > 10 mm induration and erythema, and MIF production was demonstrable *in vitro*.

Dr. LISCHNER: Now, how do you explain this?

Dr. CHILGREN: It would appear that the transfer factor is capable of 'turning on' MIF production, thereby correcting this cellular deficiency and allowing the demonstration of delayed hypersensitivity. Transfer factor and MIF are not the same thing, and how transfer factor effects this reconstitution I do not know. This reversal of anergy is temporary, lasting only about 3 weeks, implying that continued reconstitution would require repeated injections of the factor. Whether such reconstitution would favorably or adversely affect the patients' condition is unknown.

In any event, it is implied that transfer factor has functions other than sensitization of the lymphocytes, since our *in vitro* data would suggest that the patients' lymphocytes were already sensitized.

Dr. LISCHNER: Could you tell us about how many tests of the kind you showed of the MIF were done on normal individuals, showing consistently less than 80 % migration?

Dr. CHILGREN: The production of MIF was measured by Drs. ROCKLIN and DAVID using the supernatant of antigen-stimulated human lymphocyte cultures in a guinea-pig macrophage system, similar to the method of THOR. In their hands, macrophage migration less than 80 % of the control indicates the presence of MIF in the supernatant and correlates well with the skin test reactions. They tested six persons skin test positive to *C. albicans* and eight persons skin test negative to that antigen. Cell supernatants from all of the skin test positive individuals inhibited migra-

tion to less than 80 % of the controls, with a mean migration of 74 %. In contrast, cell supernatants from all of the skin test negative individuals allowed migration greater than 80 % of the control, with a mean of 102 %. Using PPD as an antigen, the correlation between skin test and MIF was 15/16 for the positives with a mean of 73 % and 10/10 for the negatives with a mean of 96 %.

Patient III's MIF production was measured only in our laboratory. We have not yet accumulated a long series of controls, and therefore, we compared this patient's MIF production with controls within each individual experiment. In three separate determinations using *C. albicans* antigen, supernatants from cultures of this patient's lymphocytes allowed migration to a mean of 115 % of the controls, while the supernatants from skin test negative controls allowed a mean migration of 106 %, and the skin test positives a mean migration of 67 %.

DANIEL C. SHANNON (Massachusetts General Hospital): About 4 years ago, while working in Dr. FRANK AUSTIN's laboratory, we were intrigued with this same disease, and we had seven patients with the chronic moniliasis, hypoparathyroidism, et cetera, syndrome. Several years ago we studied seven patients with chronic mucocutaneous moniliasis and metabolic disease. The first three that we tested, both on skin testing and *in vitro* lymphocyte culture with *Candida* antigen, were negative, and we were quite delighted with this at first. We then repeated the studies using another antigen, this time prepared in 50 % glycerol. This antigen, both *in vitro* and by skin test, showed positive results in all seven of the patients. Thus, we discarded the idea that there might be a defect in delayed hypersensitivity in such patients.

Your patients appear to have only chronic mucocutaneous candidiasis, and I wonder if you think these are two separate syndromes or if they are varying manifestations of the same syndrome. I would like your comments on this.

Dr. CHILGREN: These patients have been tested with both forms of *C. albicans* antigen prepared by Hollister-Stier Laboratories, the so-called metabolic antigen and the *Candida* extract antigen. Under all test circumstances, these patients are skin test negative. The only way we can elicit a response from these patients is to increase the concentration of *C. albicans* antigen to a very high level, for example, to 12,000 protein nitrogen units (50 units are adequate for a normal). However, this reaction is clearly a toxic one, as demonstrated by the polymorphonuclear and eosinophilic cell infiltrate in the skin biopsy.

In answer to your second question, we have a series of eight patients with chronic mucocutaneous candidiasis. Three of these were presented here today, two of whom have an associated endocrinopathy. Another patient has very severe disease with cutaneous anergy, but in contrast to the first three, has absence of *in vitro* blastogenic response to phytohemagglutinin and *C. albicans*. While she has no endocrine disease, she does have chronic staphylococcal abscesses and chronic lung disease. The other four patients have no demonstrable immune deficit, two having concomitant endocrine disfunction. While these may well represent more than one disease, or a spectrum of a single process, I do not yet see a way to clearly separate them using clinical and immunological criteria together. Perhaps the answer will lie in the meaning of the immunological deficiencies associated with this disease.

To us, the single organism specificity of this disease, the case histories of the patients and the other immunological studies we have reported suggest that the immune deficiency is acquired and probably secondary to infection with the organism. The deficiency may well contribute to the perpetuation, severity, and progression of the disease, however.

FREDERIC M. KENNY (Children's Hospital, Pittsburgh, Pennsylvania): Did you measure circulating antibodies to parathyroid, adrenal, or thyroid tissue in any of your patients presented today?

Dr. CHILGREN: No, this has not yet been done. We have arranged to send sera to Dr. BLIZZARD for this purpose.

DERRICK LONSDALE (Cleveland Clinic): We have two patients with this disease. In one of them the serum will grow the *Monilia* very profusely, and if her serum is transferred to a normal patient, the *Candida* will grow profusely in this also.

It's interesting that both these patients have responded dramatically and very fast to the use of Diodoquin. I wonder if you can comment on this.

Dr. CHILGREN: In 1964, LOURIA and BRAYTON described the rapid decrease of *C. albicans* colonies after the organisms were incubated in normal human sera. This decrease in colonies was not observable when the organisms were incubated in serum from certain chronic mucocutaneous candidiasis patients. In addition, patient sera, when added to normal sera, inhibited this decrease in culturable organisms.

In 1967, we demonstrated that the mechanism of this reaction consists of the formation, in normal serum, of large clumps of *C. albicans* yeast and germ tube organisms. The resulting decrease in the number of viable organisms was more apparent than real, since if the clumps were disrupted, the colony count returned to normal levels. *C. albicans* in the presence of patient serum, or normal serum plus patient serum, does not form these clumps, but rather continues to grow the long germ tubes. An IgG antibody to *C. albicans* is responsible for this phenomenon. Dr. LOURIA and I agree that we are probably describing the same thing. This inhibition to clumping is present in all three of the patients described here today. I expect it is also present in the sera of the patients you describe.

Dr. LONSDALE: Can you comment on the Diodoquin?

Dr. CHILGREN: We have not used Diodoquin.

I. J. WOLF (Barnett Memorial Hospital, Paterson, New Jersey): I also want to know about treatment. How about Mycostatin? Have these patients been treated orally with Mycostatin and locally with Mycostatin?

I'm sure all practicing pediatricians see candidiasis in the diaper area and other parts of the skin, and if we don't treat them, these infants and children have candidiasis for months and months and come to you from other physicians because they are not handled properly; even though the immunology is normal, if you don't treat them adequately, they don't clear the candidiasis.

So how have these chronic patients been treated?

Dr. CHILGREN: These patients have been treated by almost every conceivable method, including Mycostatin. In fact, at last count, 14 different regimens have been tried on the first of the patients presented here. None work, except intravenous Amphotericin B, which unfortunately provided only temporary relief and produced a temporary 75 % drop in renal function and a salt-losing nephropathy.

The work we have presented today would suggest that transfer factor or histocompatible bone marrow might be used to correct the cellular immune deficiency in patients such as those presented and to allow their own immune mechanisms to control the infection. In fact, the report of BUCKLEY *et al.* suggests just this approach. Her patient with staphylococcal botryomycosis was given bone marrow and peripheral blood from her father, after the patient's disease had been brought under control with IV Amphotericin B. The disease has not returned in almost 2 years, and the patient's response to skin tests was demonstrated 6 months after the transplant. No evidence of bone marrow from the father could be demonstrated, raising the speculation that transfer factor, produced *in vivo* from the father's cells, accounted for the reversal of anergy and/or control of the disease.

I agree with Dr. WOLF that vigorous treatment of oral and diaper area candidiasis with available antimycotic agents is indicated in infants and young children. Perhaps prevention of severe candidiasis is equally important. Four of the very severe mucocutaneous candidiasis patients at our insititution were treated in infancy with prolonged courses of penicillin and tetracycline. Such treatment may be what put them into this situation.

23 *Ocular Manifestations of Immunological Deficiency Disease.* MARTIN L. SCHULKIND* and JOHN M. LITTLE*, University of Florida College of Medicine, Departments of Pediatrics and Ophthalmology, Gainesville, Fla. (introduced by Gerold L. Schiebler).

Following the finding of an unusual diffuse fine epithelial keratitis in a hypogammaglobulinemic patient who had blurred vision, a study of the ocular findings in immune deficient patients was made. Of the 8 patients studied, 6 had evidence of keratitis by slit-lamp examination. The keratitis was associated with absence of lacrimal IgA and less than the normal number of conjunctival-lymphoid follicles. Interestingly, these patients had few symptoms related to the keratitis. The two patients who had no evidence of keratitis also had normal amounts of conjunctival lymphoid tissue, and one had normal level of lacrimal IgA. These 2 patients differed from the other immunoglobulin deficient patients studied in having no significant infections for the past 2 years. One of these patients had received no gammaglobulin injections for that same interval.

In most cases of keratitis no specific bacterial pathogen was isolated and a viral etiology is being investigated. Although the periodicity of the keratitis has yet to be determined, the unique occurrence in these hypogammaglobulinemic patients is striking.

These findings indicate that the eye is an easily accessible organ in which to assay some of the body's immune functions. The lacrimal gland and secretions serve as a model secretory antibody system, the conjunctivae as a source of lymphoid tissue for immunohistological studies, and the corneal status may reflect how effectively the immune mechanisms function.

Discussion

PEARAY L. OGRA (State University of New York, Buffalo): Secretory gamma-A immunoglobulin specific for viruses such as polio, measles, influenza and others have been demonstrated in the secretions of respiratory and gastrointestinal tracts. The antibody appears to be predominantly of 11 S type gamma-A. In addition, measles virus antibody has been detected in the lacrymal secretions after measles virus infection.

Have you performed any studies to detect 'secretory piece' in the lymphoid tissue of the eye, and is the gamma-A present in the conjunctiva, is 7 S or 11 S.

MARTIN L. SCHULKIND: It was a goat antiserum to 11 S human IgA.

ALFRED L. FLORMAN (New York University Medical Center): This is a very interesting observation.

I would like to remind you that normal individuals who have had measles—either naturally acquired measles or live measles vaccine—may show the same kind of keratitis with minimal or no complaint for as long as 100 days after the onset of their measles. (JAMA *179:* 568–570 [1962].)

Do you know whether your patients had either measles vaccine or measles shortly before you made your observations?

Dr. SCHULKIND: Most of these patients were teenagers or children around 8 to 10 years of age. One of the patients was 24 years old. They had all had measles earlier in their lives—natural measles.

MARY SOUTH (Baylor University College of Medicine): This is a beautiful demonstration of the biologic significance of IgA. We have been studying this over several years' time, and we have found in our population of agammaglobulinemics only one person who was completely free of any kind of mucous surface infection, and this person did have IgA, both in the saliva, which we were studying, and in the blood.

Our other patients, who had no IgA at all in either place, did have various infections of the mucous surfaces, including a very gross purulent conjunctivitis in 4 out of 20 patients.

I wonder if you have also studied the serum IgA levels in your three patients?

Dr. SCHULKIND: The serum IgA levels in all of these patients, including the three patients without keratitis, were abnormally low. In two of the three patients having no keratitis, serum IgA was absent.

24 *An Inhibitor of C'_3 Conversion Occurring During Renal Allograft Rejection in Man and Experimental Animals.* EDMOND G. SIFRI*, ROGER E. SPITZER*, ANN E. STITZEL*, and CLARK D. WEST, University of Cincinnati College of Medicine, Children's Hospital Research Foundation, Department of Pediatrics, Cincinnati, Ohio.

The role of serum complement in allograft rejection is not well established. Recently, a substance present in the serum of 4 patients and several experimental dogs has been demonstrated which inhibits the conversion and deposition of human C'_3 by immune mechanisms. When normal human serum reacts with aggregated gamma globulin, the B antigen of C'_3 rapidly disappears. In the presence of the inhibitor, however, this disappearance of the B antigen, which parallels the breakdown of C'_3, is greatly reduced. The inhibitor has not been demonstrated in the sera of patients with glomerulonephritis, nor in the sera of normal dogs, dogs with autotransplants, or dogs during the acute phase reaction which follows a 30 % body surface burn. In man, the degree of inhibition appears to be related to the severity of the rejection episode as determined by clinical means. Kinetic studies have shown that the inhibitor reduces both the total magnitude as well as the rate of C'_3 conversion. Further, it appears that the locus of this inhibition is either at the C'_3 convertase

center (C'_{2a}) or on the C'_3 molecule itself. Whether this inhibitor functions as a protective or destructive immunologic mechanism in allograft rejection is unknown. The determination of the presence of the inhibitor, however, may prove to be a valuable laboratory means of detecting early transplant rejection before instituting a potentially dangerous therapeutic regimen.

Discussion

WILLIAM T. KNIKER (University of Arkansas Medical Center): This is a very interesting observation, and one which is exciting, considering its possible significance. You pointed out that the phenomenon appears within a day or two of a primary set graft, but is present immediately at a second set graft. In various examples of immunological diseases involving purely immediate hypersensitivity mechanisms, the phenomenon was not demonstrated.

I wonder if this factor might be manufactured or released by lymphoid cells of the cellular immunity system. To prove that this factor is a mediator of delayed hypersensitivity, you might study a transplantation model in which rejection is accomplished only by antibodies. The phenomenon that you have described should be absent in that case, if the factor is indeed related to cellular immunity.

ROGER E. SPITZER: We have not had the opportunity to try this type of model. The prospect is an intriguing one.

CHESTER ALPER (Children's Hospital, Boston, Massachusetts): We have described a normal serum protein apparently absent from the serum of a young man with increased susceptibility to infection and rapid *in vivo* conversion of C3 to C3b. We have tentatively designated this protein 'C3 stabilizer', since an infusion of normal plasma markedly decreased the C3 conversion rate in the patient for a period of about 2 ½ weeks. The 'C3 stabilizer' is heat labile, whereas the protein you describe is heat stable so that they do not appear to be the same. However, the protein you describe has similar physicochemical properties to the C3 inactivator of NELSON and his colleagues and the conglutinogen-activating factor (KAF) described by LACHMANN and MÜLLER-EBERHARD. Do you think your inhibitor could be the same as this protein?

Dr. SPITZER: I do not believe that the inhibitor that occurs during transplant rejection is KAF or NELSON's C3 inactivator. These are probably one and the same substance and act solely on bound C3. Our inhibitor has no action on bound C3, but rather blocks the activation of C3 by C3 convertase.

Dr. ALPER: Do you observe C3 conversion directly as with immunoelectrophoresis or antigen-antibody crossed electrophoresis, or do you only measure disappearance of B antigen and take that to mean conversion?

Dr. SPITZER: We can do both. Using the immunoelectrophoretic precipitin method, we are able to quantitate the conversion of C3 by the decrease in the B antigen of C3. By immunoelectrophoretic analysis or by immunoacrylamide gel electrophoretic analysis, we can see conversion products and identify them by the use of monospecific antisera to the three different antigens of C3.

25 *Physiologic Studies Following Lung Homotransplantation in Animals.* LEONARD M. LINDE, KAZUO MOMMA*, MICHAEL T. GYEPES*, SEARLE TURNER*, GARY STEVENS*, and ERIC FONKALSRUD*, University of California School of Medicine, Los Angeles, Calif.

Pulmonary and systemic vascular pressures and blood gas concentrations were measured in four open chest dogs 3 to 10 months following left lung homotransplantation (LLH). Measurements were made before and after right pulmonary artery (RPA) clamping to evaluate function of the transplanted left lung. Initial vascular pressures were normal in three, but one showed collapsed left lung and mild pulmonary hypertension. Initial systemic and main pulmonary artery pH, pCO_2, pO_2 and oxygen saturations were normal in all four and did not change with RPA clamping in the two dogs with homotransplantation within six months although pulmonary artery pressures rose slightly. In a second series, four heart catheterization and angiocardiographic studies were done in two dogs and one monkey 3 to 11 months after LLH. A balloon catheter was used to transiently occlude the RPA for measurement of transplanted lung function. Initial pulmonary and systemic vascular pressures and pO_2 were normal in one dog and did not change with RPA occlusion. In the monkey, the transplanted left lung was completely collapsed with no flow. In the remaining dog, initial PA pressure was slightly elevated and blood gases were normal. No change occurred with occlusion of the RPA. Two months later, re-study revealed marked pulmonary hypertension following RPA occlusion and inability of the left lung to diffuse O_2 although CO_2 could still be eliminated. These studies indicate progressive vascular disease and loss of respiratory function with time in the transplanted left lung in spite of continued immunosuppressive therapy.

Discussion

STANLEY GOLDSTEIN (Downstate Medical Center, Brooklyn, New York): Could you tell us what your immunosuppressive regime was? You seem to have gotten an unusually long survival time.

LEONARD M. LINDE: Yes. We used azothioprine 3 mg/kg and prednisone 1 mg/kg for 7 to 10 days after operation. This was gradually tapered after recovery to an eventual dosage of Imuran 0.25 mg/kg and prednisone 0.5 mg/kg. And that was long-term immunosuppression—actually a very simple regimen.

26 *The Induction of the Pulmonary Surfactant in the Fetal Lamb by the Administration of Corticosteroids.* ROBERT A. DE LEMOS*, DENNIS W. SHERMETA*, JOHN H. KNELSON*, ROBERT V. KOTAS* and MARY E. AVERY, Johns Hopkins School of Medicine, Baltimore, Md.

The administration of ACTH and corticosteroids to fetal lambs of 110–130 days was shown to initiate premature parturition, and to lead to survival of some lambs before one would have expected the pulmonary surfactant to appear [LIGGINS, G. C., J. Endocr. *42:* 323, 1968 and BRUMLEY *et al.*, J. clin. Invest. *46:* 863, 1967]. The possibility that the synthesis of the surfactant could be induced by treatment of the fetus with steroids was examined in ewes with twin pregnancies, in which only one twin was given steroids. Catheters were placed in the peritoneal cavity or jugular vein of the fetus and exteriorized through the maternal flank to permit injections of hydrocortisone, 50 mg/day i.p. or 20 mg/day i.v. in 4 doses. The animals were born spontaneously or delivered by c-section within 72 h. Some were still-

born, others breathed and were sacrificed. In all 6 sets of twins, the lungs of the treated animals differed from the control animals: the volume of air at a peak distending pressure was approximately double that of the controls; the volume of air at atmospheric pressure was significantly greater in the treated animals; the minimal surface tension of lung extracts was consistently lowered by treatment. Adrenal weights of the steroid-treated animals were significantly reduced. The findings suggest that hydrocortisone was responsible for the premature appearance of the pulmonary surfactant. (Supported in part by USPHS grants HD 00281-09 and AM 11218-02.)

Discussion

NICHOLAS M. NELSON (Children's Hospital Medical Center, Boston, Massachusetts): Did you look at the great alveolar cell, and did you see any changes in the osmiophilic bodies therein?

Also I'd like a clear statement as to whether you feel that it is now appropriate to use ACTH in infants with hyaline membrane disease.

ROBERT A. DE LEMOS: We did not design the experiment so that tissues could be fixed for electron microscopy. Obviously, morphologic examination is a necessary next step to determine the mechanism whereby the changes we observed have occurred.

I would not be prepared to use ACTH or corticosteriods in the treatment of babies with hyaline membrane disease. There is some scattered data on this subject. A paper by Dr. SIDNEY GELLIS some years ago mentions the use of corticosteroids in the treatment of hyaline membrane disease and suggests that the mortality might be increased.

I think there are too many potential detrimental effects of corticosteroids to even suggest that they be administered, either to the mother, to the fetus or to the newborn at this time.

We certainly observed with this particular dose of corticosteroids certain rather devastating morphologic changes in organs other than the lung. When this study is carried further, there is going to have to be careful evaluation as to the effect this alteration of the intrauterine hormonal environment has on the total fetus.

ALAN HODSON (University of Washington Medical School): I'm not quite sure I understand the differences that you are seeing in the deflation pressure volume curves between 10 and 40 cm of water. Therefore, I think the histological studies might be important.

Is there a difference in structure here, or do you really think you can explain this phenomenon on the basis of surface tension differences?

Secondly, I'm sure you have thought about the mechanisms of possible induction, by steroid hormones of enzymes related to synthesis of surfactant. I wonder if you would care to hypothesize what the pathway or pathways might be?

Dr. DE LEMOS: With respect to the first question, I think the part that concerned us at the time we made these observations is: Why, on the basis of induction of the pulmonary surfactant, should the lung volume be greater at maximal inflation? It's quite clear from previous work that we would expect the lung to retain more air during deflation if the surfactant were present. Studies done by STRANG in rabbits have shown fairly clearly that the more mature lung also contains more air at peak inflation. It could be a morphologic change; it might also be an effect secondary to surfactant, atelectasis, and recruitment. We have no data from this study to choose between these alternatives. However, it is clear that induction of the surfactant has occurred.

The question of what the possible mechanism of induction is leads to a great deal of speculation since this study was not designed to test this question. I think we can say that we have changes consistent, at least, with functional appearance of the pulmonary surfactant. It's possible that the surfactant was already present and required some structural or functional alteration. It's possible that we altered the pulmonary environment to make the surfactant more effective in some manner. It's also possible that we affected the morphologic precursors of the surfactant.

I know that the fact that we saw changes in as little as 10 to 12 hours is consistant with enzyme induction. Corticosteroids have been demonstrated to reduce enzymes in other biologic systems. Further studies are indicated in this area.

RICHARD KRAVATH (Montefiore Hospital and Medical Center, New York, New York): Since you implanted catheters only in the experimental group of animals, how did you control the effect that this in itself may have had?

Dr. DE LEMOS: We tested this in another pair of twins, and hopefully we will continue to test this. We implanted catheters in one of the twin pairs in another set of lambs and infused the diluent solution without steroids. We noted over a very long time of infusion—in this case, 6 days—no demonstrable difference between the animals.

PAUL BENKE (University of Wisconsin Medical School): Do you have any gas exchange data to see if the treated animals had a more effective exchange?

And, does phenobarbital induce surfactant activity?

Dr. DE LEMOS: We do not really have any useful functional information about the state of the lung after birth. We chose other parameters of maturation so that survival and the condition of the lung after birth was not essential.

None of these lungs were really normal. They all were more normal than the twin control, but in no case am I willing to say that the lung was normal functionally, because only one or two of the animals survived for 36 hours.

As far as phenobarbital is concerned, if in fact steroids are inducing an enzyme, then it would be intriguing to try phenobarbital, since it is quite clear that that is an effective enzyme inducer. But as I said before, although I suspect it may well be that corticosteroids are inducing an enzyme, it is also possible that some other mechanism comes into play.

We do know from scattered information done by many other workers that administering corticosteroids to the fetus causes a wide variety of developmental changes. It clearly affects the kidney. In the lamb it affects the liver in the sense that all evidence of hematopoietic function disappears—at least all morphologic evidence of hematopoietic function disappears. It has been shown to affect the brush border of the duodenum. There is some question of its role in thymic development. The overall role of steroids in the developing fetus is quite vast and, in this dose, apparently quite devastating. I think although this dose of steroids to the fetus has beneficial effects on the lung, it's also quite clear that there are devastating effects on the fetus in total.

Dr. BENKE: But you have not tried phenobarbital?

Dr. DE LEMOS: I have not tried phenobarbital.

DANIEL C. SHANNON (Massachusetts General Hospital): I wonder, first of all, if the greater volumes in treated animals at any given pressure of inflation could represent alteration of elastic tissue structure as a toxic effect of hydrocortisone, which is known to be of major importance in this area of the pressure-volume loop.

And secondly, have you tried using hydrocortisone in the subphase of your Wilhelmy balance, to see if it in any way alters the surface tension characteristics of surfactant as you extract it from these lungs?

Dr. DE LEMOS: With respect to the first question, I think it's a very real possibility. I don't know. It's something that has to be tested.

With respect to the second, no, I haven't, and I don't know whether this has been done. It may well have been done by others.

DAVID W. SMITH (University of Washington Medical School): There would be one natural situation in the human, that of anencephaly, where you not uncommonly have pituitary deficiency and secondary hypoplasia of the adrenal. Have you looked at any patients with anencephaly, who generally die shortly after birth, in terms of the lung morphology or lung surfactant. This might be a somewhat natural experiment in the human toward your basic question.

Dr. DE LEMOS: I think there are two factors—or more than two; there are multiple factors involved here.

Yes, at least one anencephalic infant has been looked at, and he was found to have normal lungs when he succumbed shortly after birth.

We're not suggesting that the absence of corticosteroids, or the absence of fetal production of corticosteroids, is going to prevent pulmonary maturation. Many anencephalic infants have prolonged gestation. It's conceivable that this has to do with the timing of maturation, that is, if the corticosteroids are present at the appropriate time, it turns on the mechanism, but if they are absent, ultimately the mechanism would be turned on anyway by something else or by itself.

Obviously, the next experiment is to attempt to adrenalectomize the animal and see if we can do the converse. Technically, this has proven difficult in the sheep, but we are in the process of trying to work it out.

There are certainly many differences between man and sheep, and I think this has to be emphasized. In man, at least up to this point, it has been assumed that steroids cross quite readily from mother to fetus, whereas in sheep, it had been assumed that there was a rather significant gradient against transport of steroids from mother to fetus.

We now find that no such gradient exists in the fetal lamb, that is, there isn't any real gradient between mother and fetus, but there are, perhaps, other factors interfering with steroid transport.

A great deal needs to be learned about maternal-fetal steroid relations before we could make any hypothesis to try to go from this study in sheep (an animal with a five-layered placenta and many protein differences between mother and fetus) to man (an animal with a three-layered placenta with much easier exchange of protein across the placental barrier).

Therefore, there are many possibilities and little evidence. I don't know whether this information has any applicability to man at all. I think it just opens the door for, really, hundreds of investigations.

27 *The Effect of Fluorescent Light on the Toxicity of Bilirubin (in Vitro Studies).* NASROLLAH HAKAMI*, ROBERT P. IGO and NATHAN J. SMITH, University of Washington School of Medicine, Seattle, Wash.

Phototherapy is being used to prevent hyperbilirubinemia. These studies were designed to determine if photodecomposition products of bilirubin are toxic and whether they bind with albumin. The toxicity of photodecomposition products were determined by their inhibitory effect on mitochondrial NADH-oxidase which is shown to be strongly inhibited by bilirubin. It was found that as a result of exposure to light, both free and albumin bound bilirubin are successively decomposed to green (biliverdin) and yellow compounds. At a final concentration of 1.6×10^{-5} M, both bilirubin and biliverdin inhibit NADH-oxidase activity by 50 % while yellow compounds have no inhibitory effect up to a concentration of 3.2×10^{-5} M and only minimal inhibition at concentration of 6.4×10^{-5} M. Inhibition of NADH-oxidase by bilirubin and biliverdin could be reversed by addition of fresh human serum to the reaction mixture. However, at a serum concentration where 100 % correction of bilirubin toxicity could be achieved only 50 % correction of enzyme inhibition by biliverdin was observed, suggesting that biliverdin binds less with albumin as compared to bilirubin. The binding of biliverdin to serum albumin was also demonstrated by cellulose acetate electrophoresis. At pH 7.4, biliverdin incubated with serum migrates with albumin fraction, while free biliverdin migrates considerably faster. No albumin binding of yellow compounds could be demonstrated by Gel filtration chromatography. These results suggest that early products of bilirubin photo-oxidation (biliverdin) are toxic and bind with albumin, while later products (yellow compound) are non-toxic and do not bind with albumin.

Discussion

GERARD B. ODELL (Johns Hopkins University School of Medicine): When you did the photooxidation of your bilirubin and then subsequently electrophoresed it, did you look for protein binding by electrophoresing it at pH 7.4 rather than 8.6?

And, have you tried the photooxidation in the Petri dish in which you have an equimolar amount of bilirubin and albumin, and then study the ultrafilterability of your 4-hour oxidation product?

NASROLLAH HAKAMI: We have electrophoresed the product of bilirubin photooxidation at pH 7.4, and find very similar results to pH 8.6.

With respect to your second question, when albumin-bound bilirubin is exposed to light, qualitatively the same changes in color and spectrum occur although at a much slower rate. We have not studied the ultrafilterability of these pigments prepared as you described.

PAUL BENKE (University of Wisconsin Medical School): In addition to blocking NADH oxidase, bilirubin also uncouples oxidative phosphorylation. I wonder if you have any idea which effect is more important in the production of central nervous system toxicity.

Dr. HAKAMI: We have not studied the effect of these products on oxidative phosphorylation. We chose to study this enzyme because it is inhibited in a predictable way by a group of chemicals that have in common a carbamide group and lipid solubility. Bilirubin is one of this group of chemicals. We do not equate this inhibition with neurotoxicity. However, all of this group of compounds, which inhibit $NADH_2$ oxidase,

such as, Amytal, phenobarbital, Sedormid, etc., are central nervous system depressants.

THOMAS R. C. SISSON (St. Christopher's Hospital for Children, Temple University School of Medicine): Would you suggest from this work that intermittent irradiation of bilirubin *in vivo* might indeed be rather more hazardous to the jaundiced patient than constant irradiation? If biliverdin is, after all, an undesirable initial product of bilirubin photodecomposition, it seems that prolonged irradiation would result in other pigments than biliverdin, pigments that do not have the inhibitory effect you have found biliverdin to produce.

Dr. HAKAMI: We have considered this possibility. However, we have not done any *in vivo* studies to clarify this problem.

JOHN T. WILSON (Children's Hospital, San Francisco, California): Phenobarbital also has been used to decrease bilirubin levels in blood of infants, and, in some instances, phenobarbital and light have been used together in the neonatal period. Do you have any data, or would you care to comment, on what happens to the breakdown products of bilirubin when infants are exposed to both light and phenobarbital?

Dr. HAKAMI: We don't have any data. We have not used the combination of phenobarbital and light therapy in infants.

JEROLD F. LUCEY (University of Vermont College of Medicine): Have you had any opportunity to study the serum from infants treated with phototherapy? We have looked into the problem of whether biliverdin actually occurs in human newborn infants who have received phototherapy, and, using two different methods, we have been unable to demonstrate the presence of biliverdin in the serum.

Dr. HAKAMI: To date, we have looked for the early products of bilirubin photooxidation in the serum of only one infant and have noted some increase in optical density between 600 and 700 mμ. More studies are being undertaken to substantiate this further.

AUDREY K. BROWN (University of Georgia Medical School): Did you do these experiments only in air, coupled with light, or did you do them in a nitrogen atmosphere as well? One can certainly get the initial conversion just by exposing bilirubin to air.

The other question I had was in relation to your attributing the toxicity to the products. Were these the dialyzed, water-soluble products?

Dr. HAKAMI: We had some previous experience, which showed that molecular oxygen is required for complete photooxidation of bilirubin to proceed to yellow compounds, and as a result of this experience, we exposed small aliquots of bilirubin in covered Petri dishes. This facilitates the availability of the oxygen. Exposure of bilirubin to light in the absence of oxygen results only in biliverdin. Similarly, in the presence of oxygen and absence of light, only biliverdin is produced. Therefore, for complete photooxidation of bilirubin, both oxygen and light is necessary.

In answer to the second question, our studies suggest that the 4-hour product of bilirubin photooxidation contains biliverdin, which is more polar, and therefore more water soluble than bilirubin. This product behaves the same as crystallized biliverdin in terms of inhibition of $NADH_2$-oxidase and protein binding.

PAUL SCHULTZ (Children's Hospital, Philadelphia, Pennsylvania): Recent work by Drs. SILBERBERG, JOHNSON, and SCHUTTA has corroborated your work, using tissue culture of rat cerebellum as the experimental model. When bilirubin is incorporated into the medium, characteristic neurotoxic changes occur. However, when these bilirubin-containing media are exposed to light, the characteristic neurotoxic changes are not found, either by routine microscopy or ultrastructurally.

28 *Repression of Erythrocyte Antigen Development by Antibodies, Following Intrauterine Transfusions.* FLOSSIE COHEN and WOLF W. ZUELZER, Child Research Center of Michigan, Wayne State University School of Medicine Department of Pediatrics, Detroit, Mich.

In an infant with Rh hemolytic disease who had received 6 intrauterine transfusions, serial studies of the red cell population by agglutination, antiglobulin test (AGT) and fluorescent antibody method (FAM), permitted discrimination between the infant's own group A (A_1) R_o cells and the transfused group 0 Rh neg cells. At 7 weeks of age a minor population of ± 5 % of weakly reacting group A cells was demonstrated by their reaction with anti-A, B, but none of the cells showed any R_o (D) reactivity. At this point the AGT was negative despite free R_o) (D) antibody in the plasma. The true phenotype ('strong' A_1) of the group A cells was first established at 9 weeks of age when roughly 10 % of the circulating red cells were the infant's own. At this same time however, only a few cells gave a weak R_o (i.e. D^u-like) reaction, and the AGT remained negative. Even when (at 19 weeks) 100 % of the cells were the infant's own, the Rh reaction remained very weak, although eventually (at 9 months) they proved to be strongly R_o, the true phenotype. As the cause of the weak R_o (D) reactivity, steric hindrance was ruled out, by the consistently negative direct AGT and by experiments done when the cells had become fully reactive. Similarly, a dilution effect as a result of the intrauterine transfusions was excluded by the temporary persistence of weak R_o (D) reactivity when 100 % of the cells were the infant's own and by experiments with artificial mixtures of the fully reactive patient's cells and Rh negative cells. This represents to our knowledge, the first known example of repression of antigen formation in a growing cell population by its specific antibody. (Supported by NIH Grant No. 00505.)

Discussion

JOHN M. BOWMAN (University of Manitoba, Winnipeg, Manitoba, Canada): Early in our fetal transfusion series, we had a fetus who was given three fetal transfusions at a somewhat later period, starting at 28-weeks' gestation. The baby, a boy, was born with a cord hemoglobin of 17 g/100 ml, and was Coombs' negative, that is, all the red cells in this baby's circulation were donor in origin. He did not require any exchange transfusions after delivery, but did develop the type of anemia that Dr. COHEN has just described. At 10 weeks of age, with the first appearance of group A red cells, the baby reverted from Coombs' negative to Coombs' positive. The minor red cell population at 10 weeks of age had a well developed Rh antigen.

FLOSSIE COHEN: If I may remind you, this baby received six intrauterine transfusions from 23 to 33 weeks gestation and had none of her own red cells in the circulation until roughly 12 weeks after birth. This would indicate a very prolonged period of marrow suppression, probably down to a stage in erythropoietic differentiation (I am not sure how far back that is) be-

fore the Rh antigen is first differentiated. When subsequent marrow regeneration was resumed, Rh antigenic differentiation had to occur in the presence of considerable Rh antibody, and there was a suppression of the Rh antigen for at least as long as the antibody was present (5 months). I don't believe that one would expect these exact same findings in infants receiving two or three intrauterine transfusions with shorter periods of marrow suppression.

Dr. BOWMAN: Our baby had a lot of free antibody up to 10 weeks, and I do agree with you that the fetal transfusions were started later, but from zero reticulocyte counts and from the fact that the baby's total bilirubin never exceeded 6.5 mg/100 ml, there was for some period total suppression of this baby's marrow.

Dr. COHEN: Even though this is one complete though isolated report, we have had the opportunity to observe another such baby who received six intrauterine transfusions and whose erythrocytes had only A reactivity and no Rh reactivity at 6 weeks, but who later was quite clearly Rh positive.

MAX D. COOPER (University of Alabama, Birmingham): Could you tell us what was going on in the bone marrow at this time, and did you look for these antigens on cells of the bone marrow?

For example, if there was circulating antibody to RO (D) antigen, RO (D) cells in the peripheral blood could have been swept from the circulation by reticuloendothelial system clearance.

Since these antigens are present at an early stage of differentiation, search for RO (D) cells in the bone marrow might better reflect their production of this antigen or lack of it.

Dr. COHEN: We saw the baby for the first time at 7 weeks, when there was less than 1 % of reticulocytes in the peripheral blood, even though the bone marrow showed both a myeloid and erythroid hyperplasia. These findings initially suggested to us an active clearance of the infant's Rh positive erythrocytes soon after or just before their exit from the bone marrow as the likely mechanism of the anemia. Later, however, this interpretation proved incorrect, when subsequent examinations revealed progressively increasing numbers of the infant's own erythrocytes (identified by their A reactivity) surviving in the circulation in the presence of large amounts of Rh antibody and with a consistently negative direct antiglobulin reaction.

29 *Total Extracorporeal Support ('Artificial Placenta') in Term Lamb Fetuses for Periods up to 28 Hours.* WARREN M. ZAPOL*, THEODOR KOLOBOW*, JOSEPH E. PIERCE* and ROBERT L. BOWMAN*, National Institutes of Health, Bethesda, Md. (introduced by Gordon Avery).

In certain medical conditions involving the neonate, total respiratory support of the subject would be therapeutically desirable. The study of fetal pharmacology and physiology would also be facilitated by isolated extracorporeal perfusion. While developing such a support system we have carried out prolonged total fetal lamb perfusion utilizing our constant volume silicone membrane oxygenator. Lamb fetuses (3–4.2kg) obtained by caesarian section were cannulated via both umbilical arteries retrograde into the abdominal aorta with thin walled, non-kinking polyurethane cannulae (0.082″ OD., 0.010″ wall thickness). Blood was gravity drained into a closed reservoir and pumped at rates of 80–265 cc/kg/min through a 0.4 m^2 membrane oxygenator into the umbilical vein. Extracorporeal volume was 240 cc. Fetuses were immersed in a synthetic amniotic bath at 40°C; glucose, heparin and antibiotics were continuously infused. Perfused fetuses exhibited spontaneous physical activity throughout perfusions, sucking, swallowing and reacting to painful stimuli. Aortic pressure in term fetuses averaged 110/60 mm Hg. Blood pH, pO_2 and pCO_2 were maintained within normal fetal limits. Plasma free hemoglobin fell from 80 mg % (average) to 30 mg % within 12 h; blood lactate fell from 50 to 30 mg % within 6 h. Fetal O_2 consumption (Van Slyke determination) ranged from 5–9.5 cc/kg/min and CO_2 production ranged from 5.5–10 cc/kg/min. Fetal perfusions and metabolic studies have been performed with this system for up to 28 h. Prolonged total or partial respiratory support of the premature infant is supported by such favorable results.

Discussion

DONALD V. EITZMAN (University of Florida College of Medicine): What are the blood pressures you get on your lamb fetuses before you put them on the perfusion?

WARREN M. ZAPOL: Heparinized lamb fetuses bleed if their skin is traumatized; we, therefore, do not perform additional cutdowns to monitor blood pressure. Clamping the umbilical arterial outflow for 2 or 3 sec allows us to measure a pressure which is about 85/50 mm Hg in healthy 3-kg fetuses.

Dr. EITZMAN: I would guess from other experiences that they would be somewhere around a systolic pressure of 65 to 75 mm Hg, and the pressures you had listed here in the abstract were 110; is that correct?

Dr. ZAPOL: Instantaneous umbilical arterial clamp pressure is very nearly a function of pO_2, as has been shown by the work of GEOFFREY DAWES. The fetus appears to regulate his arterial pressure well, in spite of the fact that our placenta takes one-half of normal biologic placental flow.

Dr. EITZMAN: Well, there is also the possibility that some sort of pressor substance, or else something that might change the resistance, could be released or activated by the perfusion apparatus that you are using.

A. I. MURDOCK (Hospital for Sick Children, Toronto, Ontario, Canada): Dr. WALTER ZINGG of the Hospital for Sick Children has developed a membrane oxygenator. One of the difficulties encountered in applying the oxygenator to newborn infants, in unresponsive respiratory failure, has been the small bore of the catheters that must be used.

What is the minimal catheter size that you can use with your oxygenator that will permit a blood flow of 150 ml/min?

Dr. ZAPOL: Using our presently designed catheter, which is 0.082 inches I. D., 0.010 inches wall thickness, we obtain a blood flow of about 75 ml/kg/min from two umbilical arteries with no arterial pressure, and 30 cm of gravity drainage. With a mean arterial pressure of 70 mm Hg, we can achieve flows of 400 ml/kg/min.

GEORGE C. EMMANOUILIDES (UCLA-Harbor General Hospital):

In the initial portion of the film, it appeared that the fetus was making regular respiratory movements. Since you have so nicely controlled the blood gases and pH in these fetuses, could you tell us if you have any data regarding the cause of such respiratory movements. Is it hypoxia or acidosis, or both combined, which produces such breathing movements?

Dr. ZAPOL: Using a membrane lung, you can separate oxygen transfer into the blood from carbon dioxide transfer out of the blood. Keeping pH and pCO_2 constant and normal, lowering pO_2 will induce major fetal respiratory efforts in 3-kg fetuses.

Isolated hypoxia will start and keep a fetus breathing, but by increasing his pO_2, you can stop the motions. Acidosis or hypercapnia without hypoxia will not make a fetus begin respiratory motions.

RICHARD E. BEHRMAN (University of Illinois College of Medicine): Can you tell us whether in any of your experiments you delivered an infant after perfusion who survived?

Dr. ZAPOL: We have perfused fetuses in good condition for up to 55 h. Since presentation we have delivered a 3-kg fetus after 4 h of total perfusion, and she is a long-term survivor.

30 *Immunopathologic Studies of Insulin-Resistant Diabetes Mellitus.* W. PAGE FAULK*, COL. EDWARD J. TOMSOVIC*, and H. HUGH FUDENBERG*, University of California Medical Center and Letterman General Hospital, San Francisco, Calif. (introduced by Melvin M. Grumbach).

Anti-insulin antibodies bind I^{131}-labeled insulin *in vivo*, but the biologic ramifications of the resultant immune complexes are unknown. An 8-year-old white male insulin-resistant diabetic was diagnosed and treated with injectable insulin since age 3 years. Marked hepatosplenomegaly and lymphadenopathy were noted at age 3 years 2 months, and Coombs' positive hemolytic anemia at age 7 years 7 months. Lymph node biopsies and marrow aspirations revealed no lymphoproliferative disease. High titer insulin-I^{131} binding and hemagglutinating antibodies against beef and pork insulins have persisted for 5 years. He has developed normally. The child's mother has ulcerative colitis, but immunopathologic studies on the family showed no significant abnormalities. Evaluation of immunologic competence proved the patient to be intact for both cellular and humoral immunity. Molecular sieve and ion-exchange chromatograph studies of the patient's anti-insulin antibody revealed a 7S immunoglobulin G of both fast and slow electrophoretic mobilities. The molecule was composed of light chains of only lambda specificity. The protein responsible for the child's Coombs' positivity was eluted from his erythrocytes and found to be biochemically and antigenically identical to the serum antibody. Anaphylactic properties of the antibody were studied, and anti-insulin immunoglobulin producing plasma cells were identified in the child's bone marrow.

The data show that the patient's monoclonal anti-insulin antibody mediates both immunopathologic reactions of hemolysis and insulin-resistance.

Discussion

WILLIAM T. KNIKER (University of Arkansas Medical Center): It is generally acknowledged that a very sensitive way to detect chronic circulation of soluble circulating antigen-antibody complexes is to find granular deposits of antibody, antigen, and complement along glomerular basement membranes by immunofluorescence. Do patients with brittle diabetes, in whom large amounts of antigen (insulin) and antibody are believed to circulate in a complex form, have granular deposits of gamma globulin, insulin, and complement along their glomerular basement membranes?

W. PAGE FAULK: We haven't thought that it was justified to do a renal biopsy on this child.

However, there are some investigators using a modified form of immunofluorescence (BERNS, A. W. *et al.*, Diabetes *11:* 308 [1962]) who say that they find insulin in the glomerulus of rather severe adult diabetics. We haven't had an opportunity to test that hypothesis as yet.

ROBERT E. GREENBERG (Stanford University School of Medicine): The assumption here that a state of insulin resistance is present, I think, is subject to very significant question. For example, in every diabetic who receives insulin for any given period of time, antibodies are demonstrable, and I think it has been pretty well shown that antibody titers that do not bind more than 60 μunits/ml cannot clearly be shown to be associated clinically with insulin resistance.

Furthermore, everyone recognizes that a dose of insulin that is arbitrarily high is not, in and of itself, a very justifiable basis to claim insulin resistance.

Thus, what really solid evidence do you have on which you are claiming a state of insulin resistance?

And, have you tried to compare the antibodies present in every diabetic with the antibodies recoverable from this patient?

Dr. FAULK: It is said that insulin resistance in adults is defined as requiring 200 units of insulin per day. This figure was accepted because one is capable of abstracting 200 units of insulin from a normal adult pancreas. This child was taking 100 units of insulin when he was 3 years old: this is rather solid evidence of insulin resistance.

Dr. GREENBERG: I was struck by the relatively low binding capacity, in view of the binding capacities demonstrable in almost every child with diabetes.

Dr. FAULK: No, these titers are not low. Done by the classic method of BERSON and YALOW, they might be considered low. These titers were determined by the hydrodynamic flow technique using bovine or porcine insulin ^{131}I, as described by FELDMAN *et al.* (Amer. J. Med. *35:* 411 [1963]). Diabetics who are not insulin-resistant tend to have titers less than 1 as done by this method. This child's titer was 9.

I would say that this child does in fact have severe insulin resistance.

The case in point has been shown to share certain features of soluble-complex disease. Was this unique to this child, or was this a general phenomenon in the treatment of diabetes? I might say that up to this point we have studied several cases. Of these cases more than half were insulin resistant by the standard criteria. All of the insulin resistant patients had significant amounts of soluble complexes. None of the patients who were all right on 30 or 40 units of insulin per 24 h had significant evidence of soluble complexes.

I think there is, however—at this early phase it is hazardous to say so—a direct relation between soluble complexes and insulin resistance, or at least the complications of insulin resistance.

PAUL BENKE (University of Wisconsin Medical School): Hw can you be so sure that this patient does not have a lymphoproliferative disorder?

And also, in view of elevated lambda protein in serum, does this patient have Bence-Jones protein in his urine?

Dr. FAULK: He does not have Bence-Jones protein in his urine.

There have been several articles saying that red cell antibodies (which were the easiest ones to study) are

very often of only lambda- or kappa-chain specificity. The last report of this was LEDDY *et al.*, J. exp. Med. *121:* 1 [1965].

This child has had lymphadenopathy for 7 years with normal marrow and lymph node studies. These do not support a diagnosis of lymphoproliferative disease.

E. RICHARD STIEHM (University of Wisconsin Medical School): You showed us that the eluate reacts with insulin coated red cells, gamma G. Did you use red cells that were not coated with insulin, and show that they did not react?

Dr. FAULK: Yes. We ran them through a complete red cell panel, and they did not react.

JOHN B. ROBBINS (Albert Einstein College of Medicine): I don't understand your assumption that the antibodies that are causing the hemolysis are anti-insulin antibodies.

Dr. FAULK: Red cells do not recognize his antibody. When one puts insulin on red cells by means of tannic acid or chromic chloride, his antibody then recognizes the insulin-coated red cells.

Another proof of specificity is to take his antibody and measure it *in vitro* by means of radioautography with ^{131}I-insulin. This is an anti-insulin antibody. It is not an anti-red-cell antibody.

Dr. ROBBINS: Then I don't understand your assumption that the hemolytic anemia is due to antibodies to insulin. Isn't that what you concluded?

Dr. FAULK: His hemolysis could be caused for two reasons. One is that if he has antigen-antibody complexes on his red cells, all he needs is complement, and he has a classic passive immune lysis system.

Another possibility could be viewed in terms of the classic opsonization system. His red cells could be coated with soluble complexes and stimulate an immune clearance by the reticuloendothelial system.

Dr. ROBBINS: I assumed from your talk that you said that the anti-insulin antibodies, and perhaps anti-insulin antibody-insulin complexes, were the hemolytic agent. You mean that you do not have direct proof for that assumption?

That is, if you take your antibody, dissociate it from insulin, coat it with insulin, do you have proof that it will adhere to a red cell?

Dr. FAULK: Yes.

Dr. KUCHMAN (McMaster University, Hamilton, Ontario, Canada): Have you tried using immunosuppressives in this patient? And if so, with what results?

Dr. FAULK: None other than prednisone, but it controls his hemolysis.

DELBERT A. FISHER (UCLA-Harbor General Hospital): Has the proportion of soluble complexes decreased on dealanated pork insulin? If so, what period of time did this require? Do this patient's plasma cells recognize dealanated insulin?

Dr. FAULK: Yes, unfortunately they do. However, the proportion of soluble complexes has decreased on the hypoallergenic insulin.

CARL H. SMITH (Cornell Medical College): Dr. CANALE and I described a syndrome a few years ago (J. Pediat. *70:* 891 [1967]) of children who presented the same picture, but not diabetes. These children showed marked lymphadenopathy of 3 to 13 years duration, the features of splenomegaly, anemia and thrombocytopenia. I am wondering whether the diabetes you have in your case has not been superimposed on the syndrome we described.

Dr. FAULK: That may well be true. Do you perhaps know if your cases had soluble complexes in their sera?

Dr. SMITH: I can't answer that.

MALCOLM H. MARTIN (Georgetown University): If I understand you correctly, you said you have another 15 children who require over 100 units of insulin.

Dr. FAULK: These are adult diabetics.

Dr. MARTIN: Oh. That changes it somewhat. To have 15 children who require over 100 units of insulin would be fairly odd in most people's experience, and I was beginning to wonder how you treated your diabetic children to get them all insulin resistant.

CHAIRMAN HOLLIDAY: Dr. FAULK, of the other 15 patients with resistant diabetes, did any of them have lymphadenopathy?

Dr. FAULK: No. It's very likely that this child has some disease that we don't know about. However, he's had it a long time.

FLOSSIE COHEN (Child Research Center of Michigan): Did you look for viruses in the enlarged lymph nodes? In support of Dr. SMITH's comments, what you describe might very well have been two seperate entities. A virus infection would explain the clinical symptomatology of enlarged lymph nodes, etc. and could in turn have rendered the erythrocytes susceptible to a nonspecific attachment of the soluble antigen-antibody complex.

Dr. FAULK: That is certainly possible. Which viruses do you have in mind?

Dr. COHEN: Quite a few. Did you look for any? Cytomegalovirus for one is very ubiquitous, and has been reported to have been associated with somewhat similar phenomena (ZUELZER *et al.*, Transfusion *6:* 438–461 [1966]).

Dr. FAULK: Yes, we have looked for that. We have actively looked for infectious agents without success. Did he have normal red cells? These were screened for enzyme defects and alterations of osmotic fragility, and they behaved as if they were perfectly normal.

Dr. COHEN: Except for a shortened survival.

Dr. FAULK: Yes, because they are coated with immune complexes.

31 *Secretion Rates of Cortisol and Aldosterone Precursors in Various Forms of Congenital Adrenal Hyperplasia.* MARIA I. NEW and MARY P. SEAMAN*, Cornell University Medical College, New York, N.Y.

To elucidate the site of the enzyme deficiency in various forms of congenital adrenal hyperplasia, a method has been devised for the simultaneous determination of the secretion rates of cortisol and aldosterone precursors. Secretion rates of cortisol (F), 11-desoxycortisol (S), corticosterone (B), 11-desoxycorticosterone (DOC) and aldosterone (aldo) were determined in 10 normal subjects, children with simple virilizing adrenal hyperplasia (21-hydroxylase defect), hypertensive virilizing adrenal hyperplasia (11-hydroxylase defect) and a family with dexamethasone suppressible hyperaldosteronism under the following conditions: normal, low and high sodium (Na) diets, administration of metyrapone, dexamethasone and intravenous ACTH. The *mean daily normal secretion rates* were: F–7.5 mg/m^2; S–.26 mg/m^2; B–2.2 mg/m^2; DOC–.055 mg/m^2; aldo–.13 mg/m^2. Changes in dietary Na altered only aldo secretion. ACTH administration raised B and F secretion significantly. Metyrapone increased S and DOC secretion more than ACTH but decreased B, F and aldo secretion. In *21-hydroxylase defect* the secretion rates of B, F, DOC and S do not

increase appropriately with ACTH and aldo secretion shows a blunted increase with low Na diet. Secretion of B and F were below normal under all conditions. In the *11-hydroxylase defect* the secretion rates of B and F are very low and do not increase with ACTH while the secretion rates of DOC and S are 100 × normal and increase further with ACTH and metyrapone. Aldo secretion is very low and does not increase with Na deprivation. In the *syndrome of dexamethasone suppressible hyperaldosteronism* the secretion rates of F and aldo precursors are normal. Results confirm a deficiency of 21-hydroxylase in the simple form and a deficiency of 11-hydroxylase in the hypertensive form of adrenal hyperplasia and do not suggest an enzyme defect in dexamethasone suppressible hyperaldosteronism.

Discussion

DELBERT A. FISHER (UCLA-Harbor General Hospital): Was I correct in interpreting your slide as indicating that the aldosterone secretion had gone up in the 21-hydroxylase deficient subjects during salt depletion?

MARIA I. NEW: Yes. It is a puzzling finding that has been explained before by several investigators by the fact that the enzyme deficiency must be minimal, or at least not complete, because they do make some aldosterone, and they do have a capacity to increase the aldosterone when sodium is restricted.

Dr. FISHER: Dr. BARTTER and his colleagues have recently reported (J. clin. Invest. *47:* 1742 [1968]) that 21-hydroxylase deficient patients without salt wasting have normal aldosterone secretion and a normal aldosterone response to salt deprivation during suppression with glucocorticoid. When not suppressed with glucocorticoid, aldosterone secretion was excessive. These data suggested that 21-hydroxylation of progesterone in these patients was normal, and the authors postulated that separate isoenzymes might be present for 21-hydroxylation of progesterone and 17α OH-progesterone. Salt wasting patients are deficient in both isoenzymes while nonsalt wasting subjects lack only the isoenzyme for 21-hydroxylation of 17α OH-progesterone leaving their aldosterone biosynthetic pathway intact.

The explanation for the excessive aldosterone secretion when not suppressed with glucocorticoid was that a tendency to sodium loss (perhaps due to excessive progesterone or 17α OH-progesterone secretion) existed in patients whose ACTH production was not suppressed. Aldosterone secretion did not increase to super normal levels in your patients. Do you think the normal secretion during salt depletion implies intact 21-hydroxylation of progesterone? What other mechanism could explain the increase?

Dr. NEW: Dr. BARTTER and I have had long conversations about the difference between his data and mine, and I can only say that we do not find increased levels of aldosterone in the patients who are not salt wasters. In simple virilizing adrenal hyperplasia we do not find evidence for a difference in the activity of the enzymes for progesterone or 17 OH progesterone. Although DOC secretion is slightly increased, I do not find increased B or aldo secretion.

The reason for the rise in aldosterone secretion when Decadron is administered with a low sodium diet remains unexplained.

GEORGE T. BRYAN (University of Texas Medical Branch): I would like to comment on the rate of aldosterone secretion in control patients. We studied a total of 17 control patients and examined the relation of aldosterone secretion rate to body size, age, sex, duration of sodium intake, potassium intake, and the quantity of sodium intake.

We found that the age and the sex of the patient contributes significantly to the secretion rate, but, as you and others have indicated, the overwhelming influence is sodium intake.

A plot of the least squares regression analysis of these data shows that the mean aldosterone secretion rates in control patients on normal and low sodium diets is in the same range as Dr. NEW has presented.

Dr. NEW: Dr. BRYAN, I envy you not only the capacity to do this analysis, but I don't think I could draw it.

32 *Gonadotropins in Serum and Pituitary of Human Fetuses and Infants.* S. L. KAPLAN, M. M. GRUMBACH, and T. H. SHEPARD, Department of Pediatrics, University of California, San Francisco Medical Center, San Francisco, Calif. and University of Washington, Seattle, Wash.

Immunoreactive FSH was detected in sera from human fetuses (10) and in pituitary glands from fetuses (43) and infants (10). Serial dilutions of sera and pituitary homogenates showed similarity in immunologic reactivity to purified FSH (LER-869-2) and to FSH present in adult human sera. The serum concentration of FSH was 3.2–46 ng/ml in 10 fetuses with a gestational age of 97–133 days. No obvious sex difference was observed. These values are higher than those for prepubertal children (1–3 ng/ml) but comparable with those in patients with primary gonadal failure (8–20 ng/ml). The pituitary content of FSH was 3.3 ng (1.1 ng/mg) at 70 days gestation with a rise at 150 days of gestation to 5.9 μg in the female and 0.5 μg in the male. The FSH content of human pituitaries in the neonatal period varied from 118 ng (1.2 ng/mg) to 843.3 ng (8.3 ng/mg). Fetal sera and homogenates of fetal pituitaries were assayed for LH by a radioimmunologic method. LH was present as early as 90 days at a level of 60 ng. The mean peak level was 1.5 μg at 160–200 days of gestation. Immunoreactive serum LH was present from 97–133 days at a concentration of 10 ng/ml, some of which probably represents cross-reaction with HCG. In the pituitary of one anencephalic, the FSH content was 4 ng and the LH content 15 ng.

Discussion

DELBERT A. FISHER (UCLA-Harbor General Hospital): The variation in serum LH in the fetuses was quite wide. Is this accountable in part because of the high coefficient of variation from assay to assay?

The intraassay coefficient of variation for our TSH assay, for example, is about 2 %, but the interassay variation is about 20 %. I wonder if such variation might account for your observed LH variation, or do you think it is a real variation in concentration?

S. L. KAPLAN: The concentration of serum LH was in the range of 10–14 ng/ml without a wide variation. Serum FSH concentration rose from 7.5 ng/ml at 97 days of gestation to 46.0 ng/ml at 122 days of gestation.

The serum and pituitary specimens were assayed for FSH and LH at multiple dilutions in two or three different assays. Our interassay variation was 5–10 %.

The wide scatter shown in the pituitary content of FSH may be the consequence of the observed sex

difference, that is, the FSH content is higher in pituitaries from female fetuses.

Dr. FISHER: The LH concentrations at 150 days were relatively high, and in cord serum were essentially zero?

Dr. KAPLAN: The serum LH and serum FSH concentrations were high at 150 days, but undetectable at term in cord serum specimens.

Dr. FISHER: What was the lower limit of the assay?

Dr. KAPLAN: The lower limit of sensitivity of the immunoassay for FSH and LH is 1 ng/ml.

Dr. FISHER: What do you think accounts for the decrease in serum at term?

Dr. KAPLAN: These studies suggest that the regulation of pituitary release and secretion of FSH and LH changes or is altered at term or in the late gestational period.

Dr. FISHER: This you think is due to target organ feedback?

Dr. KAPLAN: Decreased secretion of FSH and LH serum may be the result of a target organ feedback mechanism stimulated by fetal gonadal hormones or placental hormones. However, it is always possible that maturational changes in the fetal hypothalamus may occur at term. Such a process may affect the sensitivity of the hypothalamus to stimulation or suppression by circulating gonadal hormones.

ROBERT E. GREENBERG (Stanford University School of Medicine): I'm still not quite clear on how to interpret the data. Are you able, or are you willing to say whether or not the change in serum levels following birth is related to alterations in the pituitary itself, or in terms of hypothalamic factors?

Dr. KAPLAN: This alteration in secretion of pituitary gonadotropins at birth is probably on the basis of hypothalamic inhibition. This may occur as a normal maturational process.

CHAIRMAN HOLLIDAY: Is there histological information to parallel this that would give you any clues?

Dr. KAPLAN: Histologic studies do confirm the presence of gonadotropins during the early gestational period. There is no data, of which I am aware, of quantitative changes in gonadotropin-secreting cells in the pituitary of the human fetus.

ALLEN ROOT (Children's Hospital, Philadelphia, Pennsylvania): Dr. KAPLAN, have you had any opportunity to quantitate the pituitary gonadotropins of youngsters with aberrations of sexual development such as intersex problems?

Dr. KAPLAN: No.

33 *Increased Urinary Glomerular Basement Membrane Products: A Measure of Renal Inflammation or Altered Metabolism.* WILLIAM T. KNIKER* and TOM PRINDIVILLE*, University of Arkansas Medical Center, Department of Pediatrics, Little Rock, Ark. (introduced by E. R. Hughes).

Increased urinary excretion of soluble glomerular basement membrane products (gbm-p) occurs in a variety of renal disorders [Sth. med. J. *61:* 1326, 1968]. These studies were designed to relate the degree of gbm excretion to type of kidney disorder and to determine whether urinary gbm-p excretion is abnormal in conditions not associated with renal disease. Twenty-four hour urine specimens from 142 subjects were concentrated and tested by immunodiffusion against sheep anti-human gbm globulin.

Patients with indolent chronic glomerulonephritis and non-glomerulitic renal disease excreted gbm-p in the range found for healthy controls. Subjects with 'pure' nephrosis or with renal allografts had moderate increases in gbm excretion. Marked increases in gbm-p, up to several hundred times normal, occurred in those with acute or chronic renal disease associated with active glomerulitis. In this last group, unique gbm-p were frequently observed. Two infants with congenital nephrosis excreted large amounts of abnormal gbm-p, while healthy family members excreted moderately increased amounts (heterozygous-partial expression?). Nine of eleven diabetic patients had moderate increases in urinary gbm-p; this finding could reflect the postulated generalized disorder of vascular basement membranes. These preliminary studies suggest that increased urinary excretion of gbm materials is a manifestation of active glomerulitis in renal disease and of altered metabolism in congenital nephrosis and diabetes mellitus.

Discussion

VINCENT RICCARDI (Massachusetts General Hospital): I noticed that your two patients with the Goodpasture syndrome had the highest amount of the gbm in the urine. Was their urine specifically tested with anti-gbm to distinguish a lung basement membrane material?

WILLIAM T. KNIKER: No.

CAROLYN PIEL (University of California San Francisco Medical Center): Have you had any chance to relate your findings to patients with only tubular disease, such as proximal tubular reabsorptive problems? We know that proteins are reabsorbed in the proximal tubule, and I wonder if this is influencing your observations.

Dr. KNIKER: No, we have not.

PHILIP CALCAGNO (Georgetown University Hospital): This is a rather pretty study and I think it raises many, many questions that this technique will be able to answer.

The first is the question of pyelonephritis. Now, I noted that you did not have any children with pyelonephritis, but one of the problems with which we are dealing clinically is to differentiate the urinary tract infection (recurrent urinary tract infection) that has renal involvement. It would seem to me that this approach could help answer such a question. Have you had any opportunity to study these children with pyelonephritis?

Secondly, in the child with acute glomerulonephritis in whom you found basement membrane deposits in the urine over a period of 1 year, did the basement membrane deposits disappear with clinical recovery?

Dr. KNIKER: You have brought out one of the most obvious groups that need testing. Right now we are collecting urine specimens from patients with pyelonephritis. We have studied two adults with mild pyelonephritis who were found not to have increased gbm excretion. Two infants with congenital urological obstruction, severe bilateral hydroureter and hydronephrosis, generalized nephritis, and azotemia both had moderate increases in gbm excretion.

In regard to acute glomerulonephritis, we studied many specimens over a period of months on individual patients. We found that the peak gbm excretion frequently was a few weeks after the onset, at a time when the renal function was improving and after the patients had diuresed. Abnormal levels of gbm products are still being excreted by some patients a year and a half after the onset of acute glomerulonephritis.

DONALD B. KAUFMAN (UCLA): I noticed that you mentioned that there were several extra antigenically different fragments in the more severely affected patients. I wondered if you studied biochemically any of these fragments, any of these glomerular basement membrane-like fragments, for basement membrane collagen, and if there were any differences that you noticed in their biochemical composition.

Dr. KNIKER: In this presentation, we did not have time to go into our immunochemical and physiochemical studies, which are still incomplete. Like others, we find an average of two 'normal' gbm products in the urine of healthy people. These products have rather low isoelectric points by electrofocusing and travel near albumin in ordinary electrophoresis. In nephritis, both experimental and clinical, additional unique products appear. Their isoelectric points are higher and their electrophoretic migration is more cathodal than is the case for the 'normal' products. We have found up to 6 gbm antigens in urine from patients with nephritis and upon digestion of purified gbm by enzymes *in vitro*. It is of interest that several of the nephritis-derived antigens and enzyme-derived antigens appear to be identical.

HERBERT ABELSON (National Cancer Institute): NAKANE and PIERCE (J. Cell Biol. *33:* 307–318 [1967]) have recently reported an innovative technique for labeling antibodies with enzymes, in particular with horseradish peroxidase. In their system, an anti-serum was produced using basement membrane fragments as antigen. The globulin from this serum was conjugated to horseradish peroxidase thereby forming enzymatically and immunologically active molecules. They were able to demonstrate localization of reaction product along the glomerular basement membrane.

A technique such as this should provide an adjunctive way to approach human glomerulopathies.

Dr. KNIKER: Yes, I think this technique could be quite useful. Ferritin has also been used by FARQUHAR and others to label nephrotoxic antibody in the study of its attachment to glomerular basement membrane.

PETER LEWIN (The Hospital for Sick Children, Toronto, Ontario, Canada).

This paper is of great interest to me, especially related to those cases with congenital nephrosis.

Rats given a single dose of aminonucleoside intravenously develop a nephrosis not unlike the classical form of lipoid nephrosis seen in childhood after an interval of about 6 days.

We investigated the prenephrotic stage in these animals (LEWIN and MOSCARELLO, Exp. mol. Path. *8:* 21 [1968]) and found that increased glycoprotein biosynthesis and liver ultrastructural changes were found as early as 6 hours after the administration of aminonucleoside and at least 24–48 hours before any renal changes were present.

The clinical data presented by you seems to complement our experimental evidence that in nephrosis, in contrast to the nephritides, we are witnessing a metabolic disorder that primarily involves glycoprotein metabolism in the liver, and only as a secondary or terminal event involves the glycoprotein of the basement membrane of the kidney glomerulus.

Dr. KNIKER: I think your work raises many possibilities; we too have considered the use of aminonucleoside to study gbm metabolism. Lathyrism as induced by BAPN would appear to be another possible model to study.

I did not really answer an earlier question concerning biochemical changes in collagen and glycoprotein in nephritis. Certainly it is true that such patients have biochemical alterations of the basement membrane that lead to ultrastructural, steric, and functional changes. Our studies clearly corroborate these observations by demonstrating that some glomerular basement membrane material has been so altered that it has been solubilized and manifests new antigenic sites.

WALTER HEYMANN (2103 Adelbert Road, Cleveland, Ohio): Have you tried at all in the preparation of the antigenic material to purify it? One could, for instance, easily separate Bowman's capsule from the glomerular tuft.

Along the same lines had you taken tubular suspensions of kidney, immunized sheep, and taken their serum for study?

Dr. KNIKER: We have not attempted to study a purified tubular material and cannot resolve the question as to how much the gbm-like material may have been of tubular origin.

Dr. HEYMANN: Have you done a study looking for any correlation between the intensity of glomerular material found in the urine and the selective proteinuria index in the urine?

Dr. KNIKER: We are presently relating the degree of selective proteinuria to the gbm excretion in a number of our patients. The question was answered in experimental situations in a report by HAWKINS and COCHRANE in *Immunology* about a year ago. They showed that selective proteinuria occurred in those animal models of nephritis where the excretion of gbm-like material was only slightly increased or normal. Whenever animals developed significant glomerulitis, associated with polymorph infiltration, gbm excretion increased to high levels and selectivity of proteinuria was lost. In our clinical studies, we can say that the degree of excretion of gbm-like materials does not correlate necessarily with the magnitude of proteinuria.

CHAIRMAN HOLLIDAY: Dr. KNIKER, did the parents who had material in the urine have any abnormal findings in the urine, in the usual sense?

Dr. KNIKER: We have not biopsied the parents and are not likely to. However, the 'healthy' parents and siblings did not have proteinuria (in 24-hour collections), hematuria, or azotemia. I might add that the diabetic patients that we reported today also did not have proteinuria, hematuria, or azotemia, suggesting that they as yet do not have significant renal disease.

34 *Topographic Distribution of Intestinal Enzymes.* ROMEO FORTIN-MAGANA*, JOHN HERBST*, RUTH HURWITZ* and NORMAN KRETCHMER, Stanford University School of Medicine, Palo Alto, Calif.

The topographic distribution of various enzymatic activities can be utilized to delineate the biological phase of the intestinal epithelial cell. The functional unit of the intestine is composed of the villus and the crypt. Proliferation is the characteristic of the cells in the crypt while differentiation is the prime biological aspect of the cells of the villus. These major characteristics of devolopment can be determined by a number of methods including the assay of particular enzymes as markers for specific cellular activities. Small bits of jejunum were cut transversely into 10 μ slices making it possible to use slices in sequence for enzymatic assay and histological identification. Thus, the activities would be related to exact topographic localization of

the enzyme [NORDSTROM, D. *et al.*, J. Histochem. Cytochem. *15:* 713]. Alkaline phosphatase, dipeptidase, invertase, and lactase were located predominantly in the villus with activities 5–40 times greater than that found in the crypt. Dihydroorotase, uridine kinase, and thymidine kinase are enzymes associated with DNA and RNA metabolism and are found with the greatest activity in the crypt. In fact, thymidine kinase was completely localized to the crypt and no activity was detectable in the villus. It is probable that the persistent activity of some enzymes originating in cells of the crypt is related to a longer half life of the protein. This topographic study of enzymatic activity in the intestine affords a clear basis for distinguishing proliferating from differentiating cells and indicates that the functional unit of intestine can serve as a lifelong model of development.

Discussion

JOHN R. ESTERLY (University of Chicago): I wonder if you could estimate from your histochemical studies what proportion of the activity of these enzymes was present in the mononuclear cells in the lamina propria, as opposed to the epithelial cells, particularly for the lysosomal enzymes.

NORMAN KRETCHMER: These are not histochemical studies, and we could not, therefore, estimate the proportion of the enzyme in those cells.

35 *Increased Systemic Oxygen Transport (SOT) Following Isovolumic Red Cell Volume (RCV) Reduction in Children with Polycythemia of Cyanotic Congenital Heart Disease (CHD).* AMNON ROSENTHAL*, LAWRENCE N. BUTTON*, DAVID G. NATHAN*, and ALEXANDER S. NADAS, Harvard Medical School, The Children's Hospital Medical Center, Department of Medicine, Boston, Mass.

Twenty patients aged 3–24 years with polycythemia due to cyanotic CHD were studied during cardiac catheterization in order to determine if SOT can be improved by a reduction of the RCV. The mean hematocrit (hct) was 74% (60–85) and arterial oxygen saturation 75% (56–80). Marked hypervolemia in the patients was solely due to increased RCV (82 ± 19 ml/kg). Plasma volume (PV) was normal (37 ± 8 cc/kg). The hct was directly proportional to the RCV ($r = 0.91$). Isovolumic reduction of RCV to 18 ± 8% of the initial RCV with fresh frozen plasma or 5% albumin by phlebotomy and volume replacement caused decreased viscosity and yield shear stress (mean of 0.103 to 0.043 dynes/cm^2 measured in four patients) and a significant drop in systemic resistence (SR) from 26 ± 10 to 15 ± 5 units ($p = \ll 0.01$). Stroke volume rose from 37 ± 14 to 53 ± 23 ml/m^2 ($p \ll 0.01$) and cardiac output (CO) from 3.4 ± 1.2 to 5.2 ± 2.0 l/min/m^2 ($p \ll 0.01$). This resulted in an increased SOT from 28 ± 10 to 35 ± 13 ml O_2/min/kg ($p \ll 0.01$). We conclude that erythrocytosis in response to chronic cyanosis provokes hct elevations and hypervolemia. The rise in blood volume is vital if an adequate CO is to be maintained in the face of increased yield shear stress and SR. Phlebotomy therapy decreases CO by reduction of blood volume, but acute isovolumic RCV reduction results in increased CO and SOT. The therapeutic potential of sustained isovolumic RCV reduction in cyanotic CHD deserves close attention.

Discussion

FRANK A. WALKER (Marquette University School of Medicine, Milwaukee, Wisconsin): For those of us who have large clinic populations with high incidences of G6-PD deficiency, this experiment is very practical and, in fact, happens accidentally when they are admitted to the hospital for other things.

We recently had a child who came in with a mild fever and was treated with aspirin, which produced a very marked and rapid hemolysis, and she stabilized about 6 g under her admission hemoglobin. In her case the results were slightly different: she went into failure.

And I do think that we have an additional question from this: what happens to the heart oxygen consumption, and how far down can we take the hematocrit and increase the amount of effort that the heart is exerting without getting into complications from this aspect as well?

AMNON ROSENTHAL: Congestive heart failure can certainly occur when there is a marked and precipitous decrease in the hemoglobin or hematocrit from normal level to the anemic state. The hyperkinetic response to anemia is said to occur when the hemoglobin concentration falls below 6–7 g/100 ml. The high output failure that occurs in anemia has been postulated to result from a fall in the peripheral vascular resistance due to the drop in arterial oxygen content and to a lesser degree to the fall in blood viscosity that occurs with a drop in the hematocrit. A similar situation may result in polycythemic patients with cyanotic congenital heart disease. In this situation, a marked decrease in the hematocrit will result in a substantial drop in blood viscosity and in turn, a fall in the peripheral vascular resistance. The increase in right to left shunting, which occurs in some of these patients, may further lower systemic arterial saturation and thus further reduce the peripheral resistance. A hyperkinetic state with high output failure may then result.

We have not measured coronary flow or myocardial oxygen consumption in our patients, but chronic anemia has been shown (Circulat. Res. *13:* 172 [1963]) to be associated with lower than normal left ventricular oxygen uptake despite a substantial increase in coronary blood flow. An increase in the hematocrit in that study resulted in a decrease of coronary blood flow, presumably due to the rise in the blood viscosity.

THOMAS K. OLIVER (University of Washington School of Medicine): Dr. ROSENTHAL indicated that you measured arterial blood gases and acid-base balance, but didn't give the results. Could you tell us about those too?

Dr. ROSENTHAL: When we started our study, we used fresh frozen plasma as replacement for the whole blood because of the obvious advantages it has in correcting many of the coagulation problems that polycythemic and cyanotic children have. A rise in CO_2 content and blood pH occurred in all the patients. The mean arterial CO_2 content rose from 21 mEq/liter to 26 mEq/liter, and the pH from 7.329 to a mean of 7.371 (P by paired t test < 0.001). We thought that at the time we were really improving peripheral perfusion. When we started using 5% albumin, however, we observed that no significant changes in CO_2 content or pH occurred. We think that the rise in CO_2 content and, thereby, the pH, is due solely to the presence of citric acid and sodium citrate in the fresh frozen plasma. The amount of citrate administered per unit of fresh frozen plasma is double that contained in a

similar volume of whole blood. The rise in pH, therefore, seems to be related to the infusion of fresh frozen plasma.

L. STEINFELD (Mount Sinai Medical School): Dr. ROSENTHAL, in your patients with the large right-to-left shunt, how did you measure your cardiac output to derive your systemic oxygen transport?

Dr. ROSENTHAL: The systemic cardiac output was measured by the use of the Fick principle utilizing a mixed venous saturation, arterial oxygen saturation, and oxygen consumption. In cases where there was an atrial shunt, we obviously used the superior vena caval sample.

LEONARD M. LINDE (UCLA Medical Center): There has been some suggestion, in humans and in animals, of some increased coagulability after bleeding in polycythemic subjects. Have you measured any of these factors, or did you see any thromboses in any of these patients?

Dr. ROSENTHAL: Various coagulation parameters were measured prior to and following the replacement of whole blood with an equal volume of fresh frozen plasma. Prior to the procedure, the fibrinogen level was 266 ± 63 mg/100 ml, factors VII–X 54 ± 11 mg/100 ml, factor V 54 ± 14 mg/100 ml, and following the plasma replacement, the fibrinogen rose to 316 ± 51 mg/100 ml, factor VII–X to 76 ± 22 mg/100 ml and factor V to 75 ± 17 mg/100 ml. The level of antihemophilic globulin was also improved by the procedure. There was no significant change in the platelet count over a 24-hour period. A striking finding was the presence of an inhibitor to plasminogen activation in the plasma of all the patients. The severity of the coagulation abnormalities appeared to be related to the degree of polycythemia. Erythrophoresis is now a routine procedure in severely polycythemic patients with cyanotic heart disease prior to any operative intervention because of the excessive bleeding that may occur in these patients when they are subjected to surgery. We have not encountered any thrombosis in our patients following erythrophoresis. It is conceivable, however, that phlebotomy without fluid replacement in the cyanotic patients would result in a decreased stroke volume, a drop in cardiac output, and cerebral anoxia. Acute reduction in total blood volume without concomitant red cell hemodilution would probably aggravate the slugging phenomenon and could possibly predispose to thrombosis.

J. L. NAIMAN (St. Christopher's Hospital for Children, Philadelphia, Pennsylvania): How long does it take before the polycythemia recurs?

Dr. ROSENTHAL: We do not have complete information on all our patients, but preliminary results indicate that the response varies substantially from patient to patient. Twenty-four hours following the acute red cell volume reduction there is a rise in the hematocrit, probably due to hemoconcentration that occurs with the collaboration of the fluid in the vascular and extravascular spaces. The hematocrit then decreases slightly over the next week and then continues to rise gradually with a return to normal level somewhere between 6 weeks and 3 months after the procedure.

36 *Lysosomal Acid Phosphatase Deficiency: A New Familial Metabolic Disorder.* HENRY L. NADLER* and THOMAS J. EGAN*, Northwestern University Medical School, Children's Memorial Hospital, Chicago, Ill. (introduced by Wayne H. Borges.)

Acid phosphatase (ACP) activity was found to be deficient in cultivated fibroblasts derived from a 3-month-old male. The patient expired at 4 months of age as had two siblings with a course characterized by vomiting, lethargy, opisthotonus and terminal bleeding. ACP activity was absent in the lysosomal fraction of homogenates of brain, liver, spleen, and kidney. Amniotic fluid cells obtained at 16 weeks in a subsequent pregnancy had no demonstrable ACP activity. The pregnancy was interrupted and no detectable levels of ACP were found in homogenates of fetal organs.

Cultivated fibroblasts from both parents (first cousins) and 5/11 additional family members had ACP levels of 2.7 ± 3 μm PNP/h/μg protein as compared to controls 6.2 ± 0.5. Heterozygotes could not be distinguished from controls in untreated lymphocytes, while significant differences were found after PHA treatment.

Time after PHA treatment			
	0 hours ACP*	α-gluc**	α-gluc/ACP
Controls (15)	5.7	3.7	1.5
Heterozygotes (6)	4.8	3.6	1.3
	56 hours ACP	α-gluc	α-gluc/ACP
Controls (15)	7.8	6.6	1.2
Heterozygotes (6)	3.3	6.6	0.5

* Acid phosphatase activity μm PNP utilized/h/μg protein.

** α-1-4 glucosidase activity μm maltose hydrolyzed/min/g protein.

These data suggest an alteration in the rate of synthesis or degradation of ACP exists in carriers of this genetic disorder. Lymphocyte stimulation may prove useful in detection of carriers of other familial metabolic disorders.

Discussion

CHAIRMAN HOLLIDAY: Did the parents have any symptoms that you could identify at all?

HENRY L. NADLER: None at all.

PAUL BENKE (University of Wisconsin Medical School): As you mentioned, acid phosphatase is a lysosomal enzyme. Was there a defect in phagocytosis or in leukocyte function?

Dr. NADLER: There were no studies done, either in the parents or in the children.

WILLIAM KRIVIT (University of Minnesota Medical School): We are reporting a similar set of circumstances in a family in the Journal of Pediatrics.

In our family, each infant had fatty changes in the liver similar to yours. In addition there was a fatty derangement of the kidneys. Did your family have similar involvement in the kidney?

Acidosis may also be present as a correlate in several metabolic disorders resulting in fatty liver disease. Did your patients have acidosis?

Do you have a marker of a disease with the absence of acid phosphatase? What is the relation of this enzyme's absence to the disease entity?

Dr. NADLER: We have no idea what the relation is between the deficiency of acid phosphatase and the

clinical manifestations of the disease. There is no increase in lipids within the kidney. There was no real acidosis, and the only suggestion at one time during the patient's course of some abnormality in terms of renal function was a diffuse aminoaciduria, which rapidly cleared.

R. BAEHNER (Children's Hospital Medical Center, Boston, Massachusetts): BAGGIOLINI, HIRSCH, and DE DUVE [J. Cell Biol. *40:* 529, 1969] have shown differences in subcellular distribution patterns for acid phosphatase, in rabbit leukocytes, using different chemical substrates. Have you assayed for acid phosphatase using substrates such as β-glycerophosphate or phenolphthalein instead of p-nitrophenylphosphate?

Dr. NADLER: We have used both of those, and essentially the same thing is found. One of the differences is that the β-glycerophosphatase is essentially confined to lysosomal fractions at all times, and there is no detectable activity in this patient using that particular method.

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