that killing of cholera vibrios in 36% of strains tested did not occur with concentrations approaching the limit of solubility of the drug in intestinal fluid (40 μg./ml.). This failure to effect killing of cholera vibrio strains within easily achieved antibiotic concentrations offers a possible explanation of the longer period of cholera vibrio excretion observed after furazolidone than after tetracycline treatment. It is of interest that comparable reductions in stool volume and duration of diarrhoea were achieved by antibacterial agents which differed significantly in their ability to terminate cholera vibrio excretion. It is possible that both agents rapidly reduce or inhibit the growth of the intestinal *V. cholerae* population and reduce the production of the cholera exotoxin responsible for the continuation of diarrhoea. However, tetracycline, which is vibriocidal, may rapidly carry this process to complete *V. cholerae* elimination, whereas furazolidone, which is not consistently vibriocidal in the concentrations probably achieved, may require host defence mechanisms to effect complete elimination of cholera vibrios.

Purging of convalescent cholera patients with magnesium sulphate has been used to detect prolonged carriers of cholera vibrios, which are not detectable by the culture of rectal swabs (Gangarosa *et al.*, 1966 ; Wallace *et al.*, 1967). Of 43 patients so purged in this study one from the control group was identified as a convalescent carrier of cholera vibrios. In the past two years five such prolonged convalescent carriers have been identified by this unit (Wallace *et al.*, 1967). Three of these had received no antibiotic treatment, one had received initial treatment with furazolidone (200 mg. every six hours) for two days only (not included in this study), and one received tetracycline (500 mg. every six hours) for two days, beginning on the fourth day of illness. Though tetracycline (4 g. total dose) clearly shortens the duration of detectable *V. cholerae* excretion in most patients it is not yet certain that antibiotics reduce the incidence of the prolonged cholera vibrio carrier state detected in about 3% of convalescent patients examined by this unit.

The large dosage of furazolidone used in this study was chosen to demonstrate maximal drug effect. The 1.2 g. schedule was as effective as the 2.4-g. schedule. It is probable that a schedule of 100 mg. every six hours for three days (1.2 g.) would be as effective as either schedule studied and might be associated with a lower incidence of vomiting, the only side-effect noted.

We wish to express our appreciation of the co-operation and assistance given by Dr. P. M. Manji, Superintendent of the Infectious Diseases Hospital, and Dr. J. B. Chatterjea, Director of the School of Tropical Medicine, Calcutta.

REFERENCES

Barua, D., and Mukherjee, A. C. (1963). *Bull. Calcutta Sch. trop. Med.*, **11**, 85.
Carpenter, C. C. J., *et al.* (1966). *Bull. Johns Hopk. Hosp.*, **118**, 216.
Chaudhuri, R. N., Neogy, K. N., Sanyal, S. N., Gupta, R. U., and Manji, P. (1968). *Lancet*, **1**, 332.
Chaudhuri, R. N., Sanyal, S. N., Neogy, K. N., Barua, D., and Manji, P. (1965). *Lancet*, **2**, 909.
Gan, K. H., and Tija, S. K. (1963). *Amer. J. Hyg.*, **77**, 184.
Gangarosa, E. J., Saghari, H., Emile, J., and Siadat, H. (1966). *Bull. Wld Hlth Org.*, **34**, 363.
Greenough, W. B., Gordon, R. S., Rosenberg, I. S., Davies, B. I., and Benenson, A. S. (1964). *Lancet*, **1**, 355.
Kobari, K. (1965). *Proceedings of Cholera Research Symposium*, edited by O. A. Bushnell and C. S. Brookhyser, p. 199. Washington.
Lewis, G. W. (1967). *Clin. Res.*, **15**, 426.
Lewis, G., and Sanyal, S. (1965). *Bull. Calcutta Sch. trop. Med.*, **13**, 40.
Lindenbaum, J., Greenough, W. B., and Islam, M. R. (1967). *Bull. Wld Hlth Org.*, **36**, 871.
Lindenbaum, J., Greenough, W. B., and Islam, M. R. (1968). *Bull. Wld Hlth Org.* In press.
Mukherjee, S., Guha, D. K., and Guha Roy, U. K. (1957). *Ann. Biochem.*, **17**, 161.
Wallace, C. K., *et al.* (1965). *Trans. roy. Soc. trop. Med. Hyg.*, **59**, 621.
Wallace, C. K., *et al.* (1967). *Lancet*, **1**, 865.
Wallace, C. K., *et al.* (1968). *Bull. Wld Hlth Org.* In press.
W.H.O. Expert Committee on Cholera (1967). *Wld Hlth Org. techn. Rep. Ser., No. 352*, Second Report, p. 18.

Immunological Aspects of Intrauterine Transfusion

W. R. JONES,* M.B., B.S., M.R.C.O.G.

Brit. med. J., 1968, 3, 280–282

Summary: Two cases of intrauterine transfusion were studied with reference to the viability and fate of donor lymphocytes. In one of the infants donor lymphocytes were found in cord blood in significant numbers, suggesting the presence of a degree of immunological tolerance. Though lymphocyte chimerism may occur after foetal transfusion, the absence of associated graft-versus-host reactions in the great majority of babies so treated implies that an immunological protection mechanism develops by at least the second half of gestation.

Cord serum immunoglobulins were normal in both cases except for the presence of IgA, presumably due to the passive transfer of donor protein.

The opportunity afforded by intrauterine transfusion for the study of foetal immunology is stressed. It is suggested that, in our present state of knowledge and in view of the small risk of graft-versus-host reaction, such studies should have precedence over efforts to remove immunologically active cells and protein from transfusion blood, especially since such efforts may impair the efficacy of the procedure.

* Department of Obstetrics and Gynaecology, University of Leeds. Present address: Department of Obstetrics and Gynaecology, University of Sydney.

Introduction

Intrauterine transfusion is now widely used in severe rhesus isoimmunization (Liley, 1963 ; Lucey and Butterfield, 1966). Interest has recently been aroused in the possible immunological effects of this procedure on the foetus (Hrushovetz, 1965 ; Githens, 1966) and in the valuable opportunity it affords for the study of foetal immune reactions (Sterzl and Silverstein, 1967 ; Jones, 1967a). This paper describes a study of two cases of intrauterine transfusion and reviews the immunological implications of the procedure.

Material and Methods

Two babies were investigated for the persistence into postnatal life of lymphocytes from donor blood. The sex of the foetus was determined before transfusion by sex chromatin analysis of amniotic fluid squames (Coulson and Scott, 1967). Opposite sex donor blood was then used for each transfusion in that particular foetus. The transfusion blood varied in age from three to four days. It was citrated and packed to a haematocrit of 60%. At delivery umbilical cord blood was collected for chromosome analysis and immunoglobulin estimation.

Lymphocytes were cultured from the cord blood for chromosome analysis by a modification of the method of Moorhead, Nowell, Mellman, Battips, and Hungerford (1960). The sex chromosome complement of about 30 metaphase spreads was determined.

Cord serum was also separated and immunoglobulin determinations were carried out by a radial diffusion method (Fahey and McKelvey, 1965), Immunoplates (Hyland) being used.

A routine acid elution slide test (Kleihauer, Braun, and Betke, 1957) was performed on cord blood erythrocytes in each case to determine the proportion of cells containing haemoglobin A.

The viability of lymphocytes in the bank blood used for foetal transfusions was also studied. Samples were taken from donor blood in both cases (two transfusions each) as well as from three bottles of sedimented blood supplied by the Regional Blood Transfusion Service for other purposes. Lymphocytes were separated and cultured in vitro with added phytohaemagglutinin (Burroughs Wellcome). Duplicate 10-ml. cultures were planted with an initial small lymphocyte concentration of 1,500 to 2,000/cu. mm. in Eagle's medium with 20% autologous plasma. Demecolcine (Colcemid) was added to the cultures after incubation for 69 hours at 37° C. and three hours later the cells were harvested. Stained smears were examined microscopically and the proportions of mitotic figures and of lymphoblasts ("transformed" lymphocytes) were determined in a total of 1,000 cells counted.

Results

Data concerning the erythrocyte and lymphocyte composition of the cord blood in the two cases after intrauterine transfusion are given in Table I. In each case more than 50% of the cord erythrocytes were of donor origin, signifying successful transfusions. However, whereas in Case 1 there were no donor lymphocytes present in cord blood, in Case 2 more than 50% were of donor sex. The last transfusion in this case had been 15 days before delivery. Viable lymphocytes had previously been found in the donor blood used in each transfusion in the two cases (see below).

TABLE I.—*Fate of Donor Lymphocytes in Two Cases of Intrauterine Transfusion*

Case No.	Gestation (weeks)	Gestational age at I.U.T. (weeks)	Age of I.U.T. Blood (days)	Sex of Baby	Cord Blood				
					% Adult R.B.C. Present (Kleihauer)	Mitotic Figures Examined	XX	XY	% Donor Lymphocytes Present
1	33	29 31	4 4	F	53	30	30	0	0
2*	34	30 32	4 3	M	83	28	16	12	57

* The baby, of birth weight 2,088 g., died at 48 hours during an exchange transfusion.

The cord serum immunoglobulin levels are shown in Table II; the detection of immunoglobulin A (IgA) in each case is noteworthy.

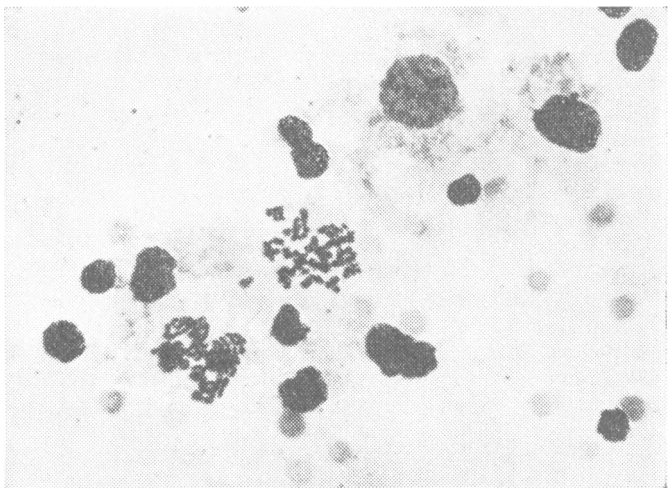
TABLE II.—*Cord Serum Immunoglobulins After Intrauterine Transfusion*

Case No.	Gestation (weeks)	Immunoglobulin (Ig) (mg./100 ml.)		
		IgG	IgM	IgA
1	33	800	12·0	9·5
2	34	720	16·5	6·5

The results of phytohaemagglutinin culture of lymphocytes from bank blood, including that used in the foetal transfusions described, are shown in Table III. Mitotic activity was detected in all samples (see Fig.).

TABLE III.—*Phytohaemagglutinin Culture of Lymphocytes from Transfusion Blood*

Specimen	Age (days)	Mitotic Figures per 1,000 Cells	Total Transformed Lymphocytes per 1,000 Cells
A (Case 1 a) ..	4	6	220
B (Case 1 b) ..	4	7	156
C (Case 2 a) ..	4	3	250
D (Case 2 b) ..	3	8	321
E	5	3	232
F	3	2	203
G	4	2	122



Transformed lymphocytes and mitotic figures in cultured three-day-old transfusion blood.

Discussion

Blood used for intrauterine foetal transfusion can reasonably be expected to contain immunological material. The small lymphocyte in peripheral blood is immunologically competent (Gowans, McGregor, Cowen, and Ford, 1962; Billingham and Silvers, 1963), and the persistence of these cells in mitotically active form for up to 22 days in bank blood has been described by Petrakis and Politis (1962). Furthermore, the present study and that of Hutchinson, Maxwell, and Turner (1967) indicate that lymphocytes can survive among packed and even washed erythrocytes for at least five days.

The fate of transfused lymphocytes has been studied in adults by injecting fresh cells from thoracic duct lymph intravenously into recipients selected for compatibility on major blood groups (Perry, Irvin, and Whang, 1967). Donor lymphocytes were found to circulate in the peripheral blood for up to nine days.

There seems little doubt that transfusion blood, even when stored for some days, contains mitotically active, and presumably immunologically competent, lymphocytes. Such lymphocytes can be expected to retain their capacity for long-term survival and multiplication in an immunologically tolerant recipient (Porter and Cooper, 1962; Elves, 1966). In the second case reported here, donor lymphocytes were present in high concentration in cord blood at a time when the majority should have been immunologically rejected. Hutchinson *et al.* (1967) found a similar lymphocytic chimerism involving donor lymphocytes in two out of six babies investigated after intrauterine transfusion; one of these babies was 16 months of age. In another case reported by Cohen, Zuelzer, Kadowaki, Thompson, and Kennedy (1965) donor lymphocytes were detected in bone marrow and in peripheral blood in an infant of 8 months. In none of these babies was there clinical evidence of any immunological abnormality.

The occurrence of graft-versus-host reaction (runt syndrome ; homologous disease) after the allogeneic grafting of immunologically competent small lymphocytes is well recognized in experimental animals (Billingham, 1959 ; Nisbet and Heslop, 1962), and may occur in totally irradiated humans who have received allogeneic bone grafts (Mathé, Amiel, and Schwarzenberg, 1964). The possibility has been raised of the occurrence of this syndrome as a sequel to intrauterine transfusion (Hrushovetz, 1965 ; Githens, 1966). It has been estimated (Githens, 1966) that approximately 20 ml. of whole blood contains enough lymphocytes to meet the requirements for the production of runting in a 1-kg. infant.

Despite the theoretical dangers of graft-versus-host reaction most authorities on intrauterine transfusion report an absence of systemic sequelae after the procedure (Liggins, 1966 ; Githens, 1966). However, some (Walker, 1967) have expressed concern at the incidence of prolonged erythropoietic depression in these babies, and the possibility should be considered that such cases might represent minor forms of runt disease.

The only recorded case of overt runt disease after intrauterine transfusion was reported by Naiman, Punnett, Destiné, and Lischner (1966). This infant had received three intrauterine transfusions between 27 and 31 weeks' gestation. At 8 weeks of age marked features of runt disease were present, and chromosome studies on peripheral blood, and on thymus obtained at necropsy two weeks later, showed the presence of donor lymphocytes.

Clinical evidence to date therefore indicates that classical graft-versus-host reaction is a rare complication of intrauterine transfusion. This suggests that in the great majority of cases the foetus is immunologically capable of eliminating donor lymphocytes. Cellular immunity in the form of allograft rejection is present in the foetal lamb (Schinckel and Ferguson, 1953) and the foetal monkey (Bangham, Cotes, Hobbs, and Tee, 1962 ; Silverstein, 1967) by mid-gestation, and it is likely that similar capabilities are present in man. Foetal lymphocytes from lymph node, spleen, and thymus, as early as the 12th week in the latter instance, display a capacity for in-vitro immune reactions which parallels that of adult lymphocytes (Jones, 1967b).

It is also significant that attempts at grafting allogeneic bone marrow and spleen cells into the foetus in the first half of gestation have been uniformly unsuccessful (Browne, 1960, 1967 ; Scott, 1964 ; Clinicopathological Conference, 1967)—further evidence of the difficulties involved in evoking tolerance even in early gestation.

In considering the effect of the introduction into the foetus of immunologically competent cells in the form of an intrauterine transfusion or a bone marrow graft it is pertinent to reflect that donor cells in the peritoneal cavity gain access to the foetal circulation via the lymphatic system (Courtice, Harding, and Steinbeck, 1953 ; MacDougal, 1958). They are therefore brought into close contact with a high concentration of foetal lymphocytes before being dispersed throughout the cardiovascular system.

Because of the risk of graft-versus-host reaction after intrauterine transfusion it has been suggested that an attempt should be made to use leucocyte-free blood for this purpose (Hathaway, Githens, Blackburn, Fulginiti, and Kempe, 1965 ; Naiman *et al.*, 1966 ; Githens, 1966). Triple-washed packed cells were used by Schwarz (1967), but in two of five cases transfused foetal death was associated with manifestations of a haemorrhagic diathesis. Schwarz drew attention to the possible elimination of coagulation factors by washing procedures and considered that this danger outweighed the unknown but probably slight risk of runt disease.

Simple methods of separating leucocytes from erythrocytes based on centrifugation and fractional aspiration are relatively inefficient and more complex methods such as dextran sedimentation and filtration probably leave the erythrocytes in less than optimum condition (Jago, 1956). Therefore, where no attempt is made to obtain lymphocyte-free blood for foetal transfusion lest it impair the efficacy of the treatment, it is important, in our present state of knowledge, to utilize these cases to examine foetal immune processes and to improve our understanding of potential immunological dangers to the recipient. Indeed, Sterzl and Silverstein (1967), commenting on the valuable opportunity afforded by intrauterine transfusion for the study of immunological mechanisms, go so far as to suggest that a suitable innocuous antigen be incorporated in donor blood to facilitate subsequent studies.

The results obtained for cord serum immunoglobulins were normal except for the detection of IgA in both cases. Similar results were obtained by Hobbs (1967) when he detected IgA in 12 babies at a mean concentration of 6 mg./100 ml. The presence of IgA in foetal serum after intrauterine transfusion almost certainly reflects donor globulin absorbed across the peritoneal membrane.

It is a pleasure to acknowledge the stimulating support of Professor J. S. Scott, who also allowed me to study one of his patients. I wish to thank Mr. H. Agar for permission to study a patient under his care, and Dr. L. A. D. Tovey, of the Regional Blood Transfusion Service, Leeds, for his co-operation. Mr. A. Coulson performed the chromosome analyses and Miss S. Rose gave valuable technical assistance. This work was undertaken during my tenure of the T. B. Walley Fellowship in Obstetrics of the University of Sydney.

REFERENCES

Bangham, D. R., Cotes, P. M., Hobbs, K. R., and Tee, D. E. H. (1962). In *Proceedings of International Symposium on Bone Marrow Therapy and Chemical Protection in Irradiated Primates*, p. 187. Rijswijk, Netherlands.
Billingham, R. E. (1959). *Science*, **130**, 947.
Billingham, R. E., and Silvers, W. K. (1963). *Ann. Rev. Microbiol.*, **17**, 531.
Browne, J. C. M. (1960). *J. Obstet. Gynaec. Brit. Emp.*, **67**, 746.
Browne, J. C. M. (1967). Quoted in P. L. Mollison's *Blood Transfusion in Clinical Medicine*, 4th ed., p. 676. Oxford.
Clinicopathological Conference (1967). *Brit. med. J.*, **2**, 819.
Cohen, F., Zuelzer, W. W., Kadowaki, J., Thompson, R., and Kennedy, D. (1965). *J. Pediat.*, **67**, 937.
Coulson, A., and Scott, J. S. (1967). Unpublished data.
Courtice, F. C., Harding, J., and Steinbeck, A. W. (1953). *Aust. J. exp. Biol. med. Sci.*, **31**, 215.
Elves, M. W. (1966). *The Lymphocytes*, p. 36. London.
Fahey, J. L., and McKelvey, E. M. (1965). *J. Immunol.*, **94**, 84.
Githens, J. H. (1966). In *Intra-uterine Transfusion and Erythroblastosis Foetalis*, Rep. 53rd Ross Conference on Pediatric Research, edited by J. F. Lucey and L. J. Butterfield, p. 104. Ross Laboratories, Columbus.
Gowans, J. L., McGregor, D. D., Cowen, D. M., and Ford, C. E. (1962). *Nature (Lond.)*, **196**, 651.
Hathaway, W. E., Githens, J. H., Blackburn, W. R., Fulginiti, V., and Kempe, C. H. (1965). *New Engl. J. Med.*, **273**, 953.
Hobbs, J. R. (1967). *Brit. med. J.*, **2**, 819.
Hrushovetz, S. B. (1965). *Canad. med. Ass. J.*, **92**, 137.
Hutchinson, D. L., Maxwell, N. G., and Turner, J. H. (1967). *Amer. J. Obstet. Gynec.*, **99**, 702.
Jago, M. (1956). *Brit. J. Haemat.*, **2**, 439.
Jones, W. R. (1967a). *Brit. med. J.*, **2**, 440.
Jones, W. R. (1967b). In preparation.
Kleihauer, E., Braun, H., and Betke, K. (1957). *Klin. Wschr.*, **35**, 637.
Liggins, G. C. (1966). In *Intra-uterine Transfusion and Erythroblastosis Foetalis*, Rep. 53rd Ross Conference on Pediatric Research, edited by J. F. Lucey and L. J. Butterfield. Ross Laboratories, Columbus.
Liley, A. W. (1963). *Brit. med. J.*, **2**, 1107.
Lucey, J. F., and Butterfield, L. J. (editors) (1966). *Intra-uterine Transfusion and Erythroblastosis Foetalis*, Rep. 53rd Ross Conference on Pediatric Research. Ross Laboratories, Columbus.
MacDougal, L. G. (1958). *Brit. med. J.*, **1**, 139.
Mathé, G., Amiel, J. L., and Schwarzenberg, L. (1964). *Ann. N.Y. Acad. Sci.*, **114**, 368.
Moorhead, P. S., Nowell, P. C., Mellman, W. J., Battips, D. M., and Hungerford, D. A. (1960). *Exp. Cell. Res.*, **20**, 613.
Naiman, J. L., Punnett, H. H., Destiné, M. L., and Lischner, H. W. (1966). *Lancet*, **2**, 590.
Nisbet, N. W., and Heslop, B. F. (1962). *Brit. med. J.*, **1**, 129, 206.
Perry, S., Irvin, G. L., and Whang, J. (1967). *Blood*, **29**, 22.
Petrakis, N. L., and Politis, G. (1962). *New Engl. J. Med.*, **267**, 286.
Porter, K. A., and Cooper, E. H. (1962). *Lancet*, **2**, 317.
Schinckel, P. G., and Ferguson, K. A. (1953). *Aust. J. biol. Sci.*, **6**, 533.
Schwarz, R. H. (1967). *Lancet*, **1**, 1103.
Scott, J. S. (1964). Unpublished data.
Silverstein, A. M. (1967). In *Comparative Aspects of Reproductive Failure*, edited by K. Benirschke, p. 392. New York.
Sterzl, J., and Silverstein, A. M. (1967). *Advanc. Immunol.*, **6**, 337.
Walker, W. W. (1967). Personal communication.