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SUCCESSFUL TREATMENT OF AN INFANT WITH SEVERE COMBINED IMMUNODEFICIENCY BY TRANSPLANTATION OF BONE MARROW CELLS FROM AN UNCLE

J. M. VOSSEN, J. DE KONING, D. W. VAN BEKKUM, K. A. DICKE, V. P. EYSVOOGEL, W. HIJMANS, ERNA VAN LOGHEM, J. RÁDL, J. J. VAN ROOD, D. VAN DER WAAY AND L. J. DOOREN

Departments of Paediatrics and Immunohaematology, University Hospital, Leiden, Isolation Ward of the J. A. Cohen Institute for Radiopathology and Protection against Radiation, Leiden, Radiobiological Institute T. N. O., Rijswijk, and Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, Netherlands

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SUMMARY

A 4½-month-old boy suffering from congenital severe combined immunodeficiency was successfully treated by transplantation of bone marrow-derived cells. His parents were cousins in the first degree. The donor was a 32-year-old maternal uncle, who was HL-A genotypically identical with the patient as was shown by serological typing and MLC. A stem cell rich fraction of the donor's bone marrow was prepared by albumin gradient centrifugation. The infant showed a full immunological reconstitution without any sign of GVH disease. Remarkably the only allotypic marker of his IgG which was different from the donor allotype remained in the serum after transplantation, and even showed an increase of its level.

The infant was nursed in strict reverse isolation and his unfavourable endogenous microflora of high potential pathogenicity was eliminated by antibiotic decontamination.

INTRODUCTION

In infants with severe combined immunodeficiency restoration of immunological capacities can be accomplished by transplantation of bone marrow cells (van Bekkum, 1972; Buckley, 1971). Successful treatment, however, has so far only been possible in cases of sibling donor-recipient pairs matched for HL-A identity and negative MLC. Even in such cases GVH disease may be a dangerous complication. Recently we described a case in which albumin gradient centrifugation of donor bone marrow cells was used to lower this risk for GVH

Correspondence: Dr. L. J. Dooren, Department of Paediatrics, University Hospital, Leiden, Netherlands.

disease in the recipient. In that case the infant showed a good take and only slight signs of GVH disease (de Koning *et al.*, 1969; Rádl *et al.*, 1972). In the following we describe the successful treatment of an infant with severe combined immunodeficiency, who was born from a consanguineous marriage. The donor was not a sibling but an HL-A genotypically identical and MLC negative uncle.

CASE REPORT

The patient, a boy, was born after a normal pregnancy. Delivery and neonatal period were uneventful. At the age of 2 weeks candidiasis of the oral cavity was noted and the boy was admitted to the University Children's Hospital, Zürich (Professor Dr W. H. Hitzig). On physical examination no other infections were found. There was a failure to thrive: the weight gain during the following 3 months was only 1230 g. An X-ray of the chest showed no thymic shadow. The peripheral blood showed a persistent lymphocytopenia (300–1900/mm^3). Lymphocytes from the peripheral blood could not be stimulated by PHA. Skin tests with candida antigen and skin tests after sensitization with DNCB and KLH were negative. Full thickness skin grafts from the parents and from an unrelated female did not show acute rejection. Further interpretation was difficult because of shrivelling of the grafts. No lymph nodes could be found in the groin on surgical exploration. Immunoglobulin G in serum was 900 mg% on admission, decreasing to 230 mg% at $2\frac{1}{2}$ months of age; immunoglobulin A was less than 1 mg%; immunoglobulin M was 17 mg%, and 26 mg% 2 months later. The boy received three diphtheria-tetanus-pertussis vaccinations. Before and after this vaccination the titre of anti-tetanus antibodies was 1:32; the titre in the serum of the mother was 1:64. On the basis of the family history (see below) and the findings mentioned above the diagnosis of severe combined immunodeficiency was made. At the age of $3\frac{1}{2}$ months the boy was transferred to the Paediatric Department, University Hospital, Leiden, for further treatment.

On admission the general condition was reasonable. The weight was 4660 g. Lymph nodes and spleen were not palpable. No tonsils could be seen. There were no infections apart from candidiasis of the skin of the groins, the genital and the perianal region. On the thighs the remnants of the 7-week-old skin grafts showed severe shrinkage. A biopsy from the skin graft from the unrelated female revealed no signs of rejection, but also no nuclear sex chromatin condensations to confirm a take. Until transplantation, $1\frac{1}{2}$ months later, the boy did not gain weight, despite the absence of infections.

LABORATORY DATA

The laboratory methods used have been described elsewhere (Rádl *et al.*, 1972). The number of small lymphocytes in the peripheral blood was between 1300 and 1900/mm^3. There was no eosinophilia. On the basis of the red blood cell morphology, haemoglobin chromatography, starch-gel electrophoresis and 1-min denaturation, thalassaemia minor was diagnosed. Bone marrow cytology was normal but no plasma cells were found. Immunofluorescence studies of the bone marrow for the presence of intracellular immunoglobulins showed only a few small lymphocytes positive for μ chains, but no cells positive for either γ, α or δ chains. There were also no positive cells visible after staining with conjugated anti-light chain sera. Total γ-globulin in serum was 350 mg%, decreasing to 200 mg% just before

transplantation. In the serum IgG was 2% and IgM 23% of the value present in pooled adult standard serum; IgA and IgD were not detectable. Mixed saliva and tears contained only free secretory piece apart from a trace of IgG of kappa type in the mixed saliva. In the serum no isohaemagglutinins and no antibodies against tetanus, diphtheria or whooping cough could be detected. Skin tests with candida antigen were negative. Lack of PHA stimulation of peripheral lymphocytes was confirmed. All findings were considered to be consistent with the earlier made diagnosis. Red cell groups, leucocyte groups and Gm, Am and Inv factors of serum immunoglobulins are shown in Table 1.

TABLE 1. Red cell groups, HL-A groups and Gm−, Am− and Inv factors of father, mother, infant (recipient) and donor

Red cell groups		
	Father	A_2, CCDee, MM, P_1+, K+, Fy^a+, S−, Cw−
	Mother	A_2, CCDee, MN, P_1+, K−, Fy^a−, S−, Cw−
	Recipient	A_2, CCDee, MN, P_1+, K−, Fy^a+, S−, Cw−, Le^a+
	Donor	AB, CcDee, NN, P_1+, K−, Fy^a−, S−, Cw−, Le^a−
HL-A groups		
	Father	HL-A 1 9/HL-A 5 /4A 4B
	Mother	HL-A Ba* Li/HL-A BB Maki/ 4B
	Recipient	HL-A 1 Li/HL-A 5 Maki/4A 4B
	Donor	HL-A 1 Li/HL-A 5 Maki /4A 4B
Gm−, Am− and Inv factors		
	Father	Gm (a−x−f+n+g−b+s−t−c^3−c^5−), Am(1+), Inv(1+a+)
	Mother	Gm (a−x−f+n−g−b+s−t−c^3−c^5−), Am(1+), Inv(1−a−)
	Recipient	Gm (a−x−f+n+g−b+s−t−c^3−c^5−), Am(1−),° Inv(1+a+)
	Donor	Gm (a−x−f+n−g−b+s−t−c^3−c^5−), Am(1+), Inv(1+a+)

° Am factor was present at 4½ months after transplantation.

FAMILY STUDIES

The boy was the fifth child of Italian parents who were cousins in the first degree (Fig. 1). Three of the four siblings, all boys, died during infancy from failure to thrive, diarrhoea and mycotic and bacterial infections. Autopsy in one case confirmed the diagnosis of severe combined immunodeficiency. The fourth sibling, also a boy, died from complications of oesophageal atresia. His lymphoid tissues appeared normal on autopsy. No other cases of immunodeficiency could be traced in the family. The parents were clinically healthy.

The blood smear and the serum immunoglobulin levels of the father were normal. In the mother thalassaemia minor was diagnosed. The levels of IgG and IgA in her serum were normal, but IgM was highly increased: five to ten-fold the level in normal adult serum. There were no paraproteins.

Because the parents were first degree cousins it was possible that family members could be found among the siblings of the parents and their children, which were not only phenotypically but also genotypically identical. Indeed of the seven family members (uncles and aunts) investigated, a 32-year-old brother of the mother living in Canada was genotypically HL-A identical with the infant, and a MLC was negative (Table 2). He showed no abnormalities on clinical and laboratory examination, except thalassaemia minor. Serum immuno-

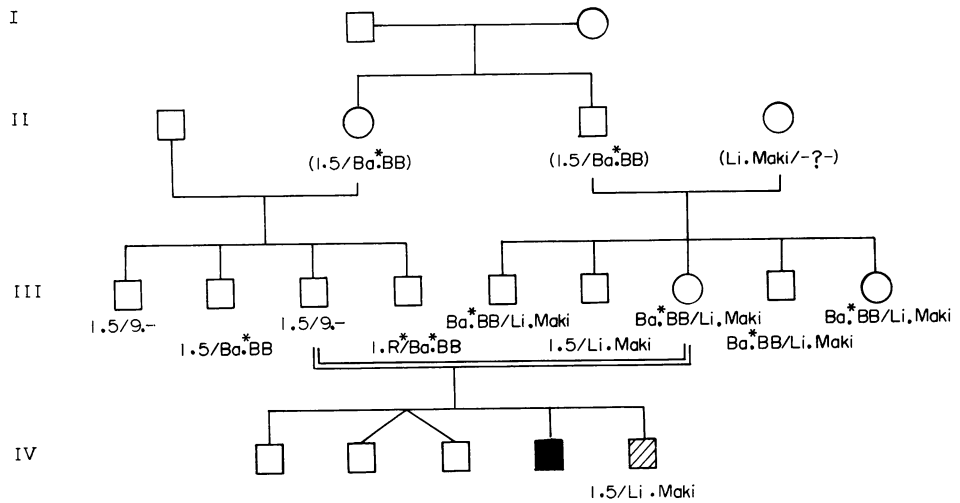

FIG. 1. Pedigree of patient's family showing inheritance of HL-A genotype. The three open squares of IV, severe combined immunodeficiency; solid square, oesophageal atresia; diagonally hatched square, propositus.

TABLE 2. Results of PHA stimulation and mixed lymphocyte culture (MLC) of the patient (P), his father (F) and his donor-uncle (U), measured by ^{14}C-thymidine incorporation

		c.p. 10 min	s.e.
PHA	P	387	45
	F	24·989	573
	U	25·168	876
MLC	P+U	0	–
	Pm+U	99	85
	P+Um	6	—
	P+F	4949	649
	Pm+F	1478	129
	P+Fm	1473	369

m = Mitomycin treated.

globulin levels were normal, as were the results of cytological and immunofluorescence studies of his bone marrow. Skin testing was negative with purified protein derivate (tuberculin) 1:1000, weakly positive with candida antigen and strongly positive with mumps antigen. Red cell groups, leucocyte groups and Gm, Am and Inv factors of serum immunoglobulins are shown in Table 1.

BONE MARROW TRANSPLANTATION

Transplantation was carried out when the boy was 4 months and 3 weeks old. A total

volume of 330 ml of bone marrow mixed with peripheral blood cells was aspirated from different sites of the iliac crest of the donor under general anaesthesia. It was necessary to remove the majority of erythrocytes (98%) from the punctate by a buffycoat method in order to obtain a nucleated cell suspension suitable to be fractionated by albumin density centrifugation. The methods used have been described elsewhere (Dicke, Lina & van Bekkum, 1970). Fraction 3, located in the 21% albumin solution of the gradient, density 1·062, yielded 9% of the original cell suspension. Since it had been found empirically that stem cell concentrates containing more than 3% of the unfractionated material are contaminated by lymphocytes, it was decided to refractionate fraction 3. For this purpose an albumin gradient was used, prepared from a 35% albumin stock solution with a slightly increased osmolarity, 375 mmol instead of 365 mmol. A fraction (3/2) was isolated containing an eight-fold concentration of *in vitro* colony-forming cells determined according to the *in vitro* culture method described elsewhere (Dicke, 1972; Müller-Bérat, 1972), which can be used as quantitative assay for haemopoietic stem cells. In the same fraction the PHA response test was negative indicating a decreased proportion of GVH active cells by at least a factor of 10. From fraction 3/2, 5×10^6 cells/kg body weight suspended in 3 ml of Tyrode's solution were injected intravenously into the child. No complications were noted in donor or recipient. One day after transplantation a blood transfusion with irradiated (2000 r) blood was given, because the blood sampling for diagnostic investigations had caused anaemia.

RESULTS

From 6 days after transplantation (AT) the boy slowly gained weight and from 19 days AT there was a steady increase in weight (Fig. 2). From 9 days AT onwards tube feeding, instituted because of general weakness, was no longer necessary. The boy now resumed quickly a normal psychomotor development. From the 24th day AT a slight periorbital oedema was noted during a few days and a very faint exanthema appeared on the trunk, gradually extending to the face and to the extremities. Repeated skin biopsies showed no histological abnormalities. When bathing with chlorhexidine 0·1%, which was part of the decontamination procedure, was discontinued the exanthema disappeared promptly. There were no other clinical signs which might suggest GVH disease. The serum-GPT level was 36 units before transplantation and decreased steadily afterwards. In the faeces epithelial cells were never detected.

The number of small lymphocytes in the peripheral blood, being lower than normal before transplantation, decreased rather sharply directly AT and started to rise from the 24th day, reaching values above 2500/mm^3 at 2 months AT. At 1 month AT quantitative PHA stimulation of peripheral lymphocytes showed a reactivity of 25% of that of a normal adult rising to 58% at 6 months AT. Skin tests with candida antigen were done at day 14 and 24 AT, giving a strongly positive reaction. Skin testing with mumps antigen on day 14 AT gave a weakly positive reaction.

A steep rise of total γ-globulin in serum was noted starting at 4 weeks AT (Fig. 2). IgM, which decreased during the first week AT to values of less than 1% of normal adult serum, started to rise sharply from the second week AT, followed by IgG and IgA at 5 and 6 weeks AT respectively (Fig. 3). Repeated immunofluorescence studies of the bone marrow showed an enormous increase in the number of IgM-, IgG- and IgA-containing cells consecutively, reaching peak numbers at roughly 10–14 days before peak levels of these immunoglobulins

in serum (Fig. 3). Sporadic cells positive for IgD were found on day 67 AT. Immunoglobulins in the secretions began to appear from three weeks AT (Fig. 3). Both types of light chains were present in normal proportions in all classes of immunoglobulins in the bone marrow cells, as well as in the serum and the secretions. The serum immunoglobulins however showed a slower electrophoretic mobility and a restricted heterogeneity in comparison with normal human serum. In addition multiple transitory and very faint M-components were detectable throughout the whole 9 months observation period AT. Estimation of IgG-subclasses in the serum by immunoelectrophoresis on day 101 AT showed a high

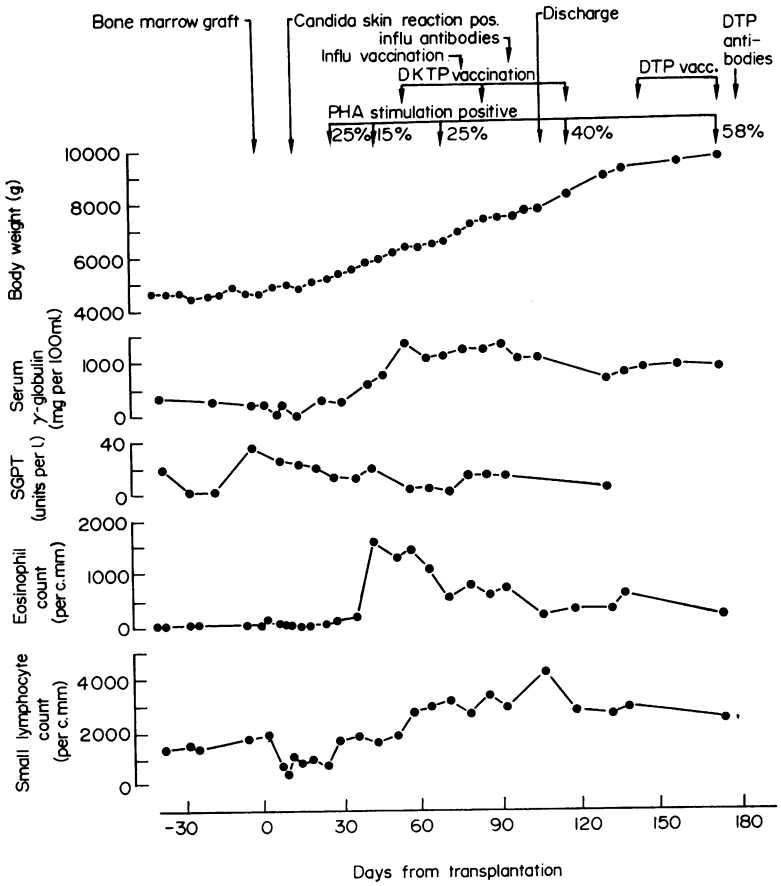

FIG. 2. Clinical course. PHA stimulation is expressed as the percentage of the average reactivity of a normal adult.

level of IgG1, a low amount of IgG2 and IgG3, and absence of IgG4, but on day 161 AT all four IgG-subclasses were already detectable. Both IgA-subclasses were then present in serum and tears. On day 267 AT IgD was detected for the first time in the serum with a level of 2·4 mg%.

The titre of anti-B isohaemagglutinin in the serum still being zero at 5½ months AT, was 1:32 at 9 months AT. On day 80 AT the boy was vaccinated with influenza vaccine (A 2'/Hong Kong/1/68 and B/Nederland/66). Eighteen days later the anti-A 2' influenza antibody

titre in the serum had risen from <9 to 480, but the anti-B influenza antibody titre did not rise significantly. After three vaccinations with diphtheria, pertussis, poliomyelitis I, II and III and tetanus antigen no rise in titres of antibodies in the serum could be detected. Following two additional vaccinations at approximately 5 and 6 months AT, high antibody titres were found: diphtheria antibodies 2·3 IU/ml, tetanus antibodies 36·20 IU/ml and poliomyelitis antibodies type I 1:2048, type II 1:512 and type III 1:256. Gm- and Inv-factors were the same in donor and recipient with one exception of Gm(n+) type in the

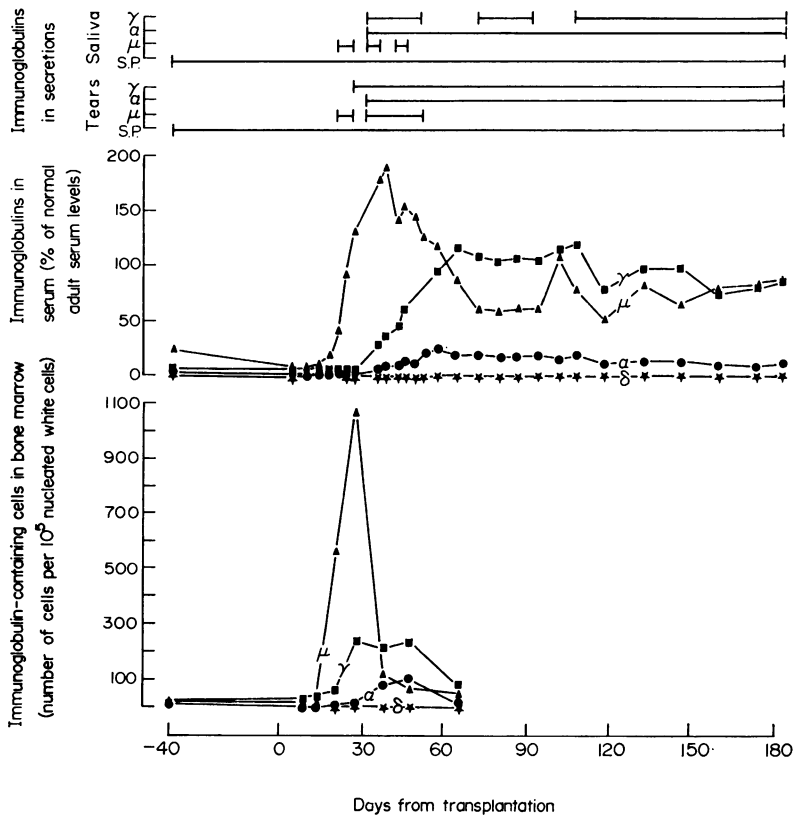

FIG. 3. Immunoglobulins in serum and secretions, immunoglobulin containing cells in bone marrow. S.P.= secretory piece.

recipient (Table 1). The titre of this factor in the infant's serum rose from 1:8 before transplantation to 1:120 at 4½ months AT. At that date the serum contained also Am-factor (IgA_2) and anti-Gm(a) antibodies. Up to 9 months AT the B-antigen of the donors red blood cells could not be detected in the infant's blood by means of the minor cell population technique.

Reverse isolation and antibiotic decontamination

The infant was nursed in a laminar cross-flow bench as described before (de Koning *et al.*, 1970). His microflora consisted of a number of potential pathogens, such as several Entero-

bacteriaceae species, *Pseudomonas* species and *Candida albicans*. Because of the unfavourable sensitivity pattern of these micro-organisms against anti-microbial drugs, it was decided to eliminate the microflora by gastrointestinal decontamination, combined with skin disinfection and repeated sterilization of the inside of the isolator (van der Waay, de Vries & Lekkerkerk, 1970a). On the basis of the results of a sensitivity test designed for this purpose (van der Waay *et al.*, 1970b) a combination of gentamycin, cephaloridin and mycostatin was chosen. Oral administration of these drugs was combined with daily bathing in a 0·1% aqueous solution of chlorhexidine. Within a week all faecal cultures were sterile and within 3 weeks micro-organisms could no longer be cultured from the oropharynx and the skin of the infant. Apart from mild nausea and vomiting, this decontamination procedure was complicated by hypocalcaemia, hypomagnesaemia, hypokalaemia and later acidosis caused by faecal loss of electrolytes. These disturbances could only be corrected by intravenous fluid therapy. When all bacterial cultures of the infant had been sterile for 24 days the antimicrobial drugs were discontinued. Three, 4 and 5 days later an anaerobic human microflora, presumably of no pathogenicity as tested in germfree lethally irradiated mice, was introduced into the rectum. This flora colonized the gastrointestinal tract within 5 days and from the seventh day onwards the level of serum electrolytes remained normal. From 25 days after this recontamination onwards two *Escherichia coli* biotypes were cultured from the faeces and other sites and from 39 days onwards *Staphylococcus albus* was also isolated. In view of the biotyping results of *Escherichia coli* (van der Waay & Kal, 1971) and of the phagotyping results of *Staphylococcus albus* (van Boven, Verhoef & Winkler, 1969) these micro-organisms were probably remnants of the original microflora. Apart from these bacteria no other micro-organisms were found until discharge, 149 days after the start of reverse isolation.

DISCUSSION

Successful immunological reconstitution in infants with severe combined immunodeficiency has until now only been accomplished by transplantation of bone marrow cells from HL-A identical MLC negative sibling donors, although more or less severe GVH disease always occurred (Buckley, 1971). Infants receiving bone marrow cells from non-identical donors invariably died from infection and/or GVH disease. Because the parents of our patient were cousins in the first degree it could be calculated that the patient had a chance of 1/30 to be HL-A identical with one or more of the seven siblings of the parents. With the help of laboratories in several countries a search was carried out with success: a brother of the mother living in Canada was HL-A genotypically identical and a MLC was negative. If an HL-A identical donor is needed for the offspring of a cousin marriage not only the sibs of the propositus and the sibs of the parents but also other available family members should be studied, like the grandparents, their sibs and the great-grandparents. Where to start first can be predicted if the number of haplotypes shared by the common grandparents is known.

The technique of bone marrow transplantation was the same as in our first successfully treated patient, using albumin gradient centrifugation (de Koning *et al.*, 1969). Whereas others using this method noted only partial or no immunological re-constitution (Levey *et al.*, 1971), we reached in this second patient again complete recovery. It should be noted that the infant showed no signs of GVH disease whatsoever. Repeated skin biopsies showed a completely normal histological picture. In three other cases where the fractionation was

performed by Dicke (1972) elsewhere, takes of the stem cell graft have been demonstrated (van Bekkum, 1972). It seems possible therefore, that the failure by others to obtain equally satisfactory results is due to a slight difference in the separation procedure. Contrary to the treatment of our first patient, this second patient did not receive a foetal thymus graft. Fractionated bone marrow cells alone seem to be sufficient for complete recovery. The findings of Hitzig *et al.* (1971) seem to point to the fact that in untreated infants a progressive involution of the thymus occurs in later stages of the disease. It remains therefore possible that thymic transplantation may be necessary in addition to bone marrow grafting when treatment is given in a later stage.

The immunological reconstitution of the infant proceeded in the same way as in our first patient. Some differences could however be noted. Immunoglobulins in the serum started to rise at about the same time after transplantation and in the same sequence, but the process was more gradual in the patient described here and less overshooting of Ig levels was noted. Also the appearance of M-components was less pronounced, and the sum of individual immunoglobulin classes showed a normal kappa : lambda ratio. Restricted heterogeneity of the immunoglobulin spectrum presently shows a gradual normalization.

As regards specific antibody production, the patient produced influenza antibodies readily after vaccination at $2\frac{1}{2}$ months AT, but no antibodies against diphtheria, pertussis, poliomyelitis and tetanus after vaccinations at circa 2, 3 and 4 months AT (Fig. 2). Specific antibody production was however very good following two additional vaccinations. Maybe the vaccination was started too early during the course of immunologic reconstitution when corresponding antibody-producing cell lines had not yet matured. The first patient showed a good response following vaccination from 3 months AT onwards, but he failed to produce poliomyelitis antibodies until more than a year AT, notably at the same time when IgA of lambda type appeared in the serum and the secretions (Rádl *et al.*, 1972). The presence of anti-Gm(a) antibodies in the serum at $4\frac{1}{2}$ months AT points to active IgM antibody production, perhaps elicited by the transfusion of (irradiated) blood shortly after transplantation. In contrast to the results in our first patient the serum of this infant showed still no anti-B isohaemagglutinin at $5\frac{1}{2}$ months AT but at 9 months AT the antibody was demonstrable in a titre of 1 : 32.

Both patients showed striking eosinophilia starting at ± 11 and ± 36 days AT respectively. This was also noted by others after transplantation of a foetal thymus in an infant suffering from Di George syndrome (Cleveland, Fagel & Brour, 1968). In our first patient we considered this as a possible sign of GVH disease, but as our present patient did not show any other sign of GVH disease, we suppose that this eosinophilia may have been induced by the appearance of immunocompetent thymus-dependent lymphocytes without relation to GVH disease (Walls *et al.*, 1971). The initial decrease in the level of circulating immunoglobulins and of the number of lymphocytes in the peripheral blood in the first week AT is unexplained. This phenomenon was also noted in other patients (de Koning *et al.*, 1969; Rubinstein, Speck & Jeannet, 1971). It could be due to the clearance of the recipient's cells by the lymphocytes of the donor, and thus be an expression of a minimal GVH reaction, which in our case did not lead to any further symptomatology.

In fact the absence of detectable symptoms of GVH, together with the findings of certain immunoglobulin production of the recipient type deserves special attention in this case, because this necessarily raises the question whether donor or recipient cells are the carriers of the reconstituted immune capacities. It is generally assumed that the grafted cells prolifer-

ate in the recipient suffering from severe combined immunodeficiency and thus substitute the lymphoid precursors which are either absent or defective in the recipient. There are reports supporting this point of view. The patient described by Harboe *et al.* (1966) produced an IgG paraprotein of donor allotype following transplantation of a foetal thymus. Immunoglobulins of Gm types which were absent in the donor but demonstrable in the recipient before transplantation were replaced in a case of Rubinstein *et al.* (1971). In our previous patient the lymphocytes of the peripheral blood showed donor karyotype after bone marrow transplantation (Rádl *et al.*, 1972). In the case reported here the γ-globulin allotypes of the recipient and the donor showed only one difference, the recipient being Gm(n) positive (Table 1). At $4\frac{1}{2}$ months AT the infant showed an approximate fifteen-fold increase of IgG_2 Gm(n+) level in the serum, as judged by the rise in inhibiting titre from 1:8 to 1:120. The latter value is within the low normal range, which indicates heterozygosity for Gm(n). This is in accordance with the phenotypes of the parents (Table 1). There is no reason to explain this finding in the recipient as a result of a technical error and it supports the idea that the IgG_2 Gm(n+) molecules were synthesized by cells of the recipient. In addition, because the donor lymphocytes carry the B antigen (Gurner & Coombs, 1958), the appearance of the complete (IgM) anti-B antibodies in the patient at 9 months AT might also be regarded as activity of the recipient cells. These findings can be interpreted in several ways. The recipient's immunoglobulin-producing clones may represent a few residual cell lines which are always present, even in cases of severe agammaglobulinaemia. Their increased activity, going parallel with the reconstitution of immune capacity after transplantation, is perhaps a result of co-operation with the donor cells. One can also go further and speculate that the other immunoglobulins are also produced by recipient cells, and even that no chimaerism is established. The function of the transplanted stem cells would then have to be viewed in another light than being a substitute for absent or defective lymphoid precursor cells. Maybe the donor cells only in some way help the defective recipient precursor cells to resume their normal function. In this connection more research should be directed to elucidate the significance of the small lymphocytes, which are positive for the μ heavy chain on fluorescence and which we consistently found in appreciable numbers in the bone marrow of these infants prior to transplantation. The situation might be even more complicated as seems to be indicated from some animal experiments. After transplantation of allogeneic bone marrow cells into irradiated rabbits Richter & Abdou (1969) demonstrated with the use of anti-allotype sera that the antibodies produced were of recipient type. The findings of others (Adler, Fishman & Dray, 1966; Bell & Dray, 1971) point to immunoglobulin production of recipient allotype by donor cells.

Nursing in strict reverse isolation (de Koning *et al.*, 1970; Vossen & van der Waay, 1972) was carried out with success. Similar results were reported by Solberg *et al.* (1971). Antibiotic decontamination (van der Waay *et al.*, 1970a; van der Waay *et al.* 1970b) resulted in complete suppression and almost complete elimination of the microflora. Only three strains of potential pathogenic micro-organisms, evidently remnants of the original microflora, could be isolated from 25 days after the anti-microbial drugs had been discontinued. In this patient decontamination caused loss of water and electrolytes leading to hypocalcaemic tetany and acidosis. Recontamination with an apathogenic selected human faecal flora resulted in complete recovery within 1 week. Antibiotic decontamination in these infants must be considered as a rather hazardous procedure and clearly deserves further detailed study. Such decontamination will be easier, or will even not be necessary at all, if the

endogenous microflora is of low potential pathogenicity. This may be the case in infants who are diagnosed and treated at an early age, and who have either not yet been in hospital or have been nursed in strict reverse isolation. Another reason for attempting the earliest possible diagnosis and treatment of these children is the possibility of development of cerebral damage by 'slow' virus infections (Bensch *et al.*, 1971; Dayan, 1971).

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