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IMMUNOLOGICAL FEATURES IN A CASE OF CHRONIC GRANULOMATOUS CANDIDIASIS AND ITS TREATMENT WITH TRANSFER FACTOR

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SUMMARY

A boy aged 6 years with severe chronic mucocutaneous candidiasis of the granulomatous variety has been treated with the dialysable transfer factor. Before the treatment he was unable to express delayed hypersensitivity. His lymphocytes were able, however, to produce migration inhibitory factor following *in vitro* challenge with soluble candida antigens, but this activation was not accompanied by a significant increase in DNA synthesis.

The treatment resulted in a temporary clinical improvement and return to normal of his immune responses. After a relapse he was, 6 months later, once more injected with transfer factor, which again proved effective.

Evidence is presented which suggests a defect of monocyte-macrophage function in this patient and the pathogenesis of candida granulomata is discussed. It is postulated that an amplifying mechanism involving lymphocyte-macrophage interaction is required for the normal expression of delayed hypersensitivity reactions and the effective elimination of *Candida albicans.*

INTRODUCTION

Immunological phenomena associated with chronic mucocutaneous candidiasis have recently been intensively studied by many workers (reviewed by Kirkpatrick, Rich & Bennett, 1971). The results have shown that there is no common immunological denominator in this disease. The most common finding, however, is defective cellular immunity, which may or may not be accompanied by failure of *in vitro* lymphocyte transformation. We have recently investigated twenty-six patients with this disease. On the basis of their cellular immune responses, we have been able to distinguish four immunological patterns and classify the patients accordingly. A detailed account of these studies will be given elsewhere (Valdimarsson *et al.*,

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1972b). In the present communication we report the immunological findings in a patient with candida granulomata and our attempts to correct his defect by injecting a dialysable extract of leucocytes from candida sensitive donors.

CASE REPORT

History

There is no family history suggestive of immune deficiency. In the first 2 years of life, the patient was immunized according to the routine schedule without any complications. He was

FIG. 1. The patient at the age of 4 years.

well until 2½ years of age when oral thrush developed. The lesions were extensive and involved the tongue, gums and cheeks. Shortly afterwards he started to have chronic respiratory infections and consolidation was found in his right lung. The mouth lesions failed to respond to Nystatin and spread to his face, fingers and toes. Since that time he had a persistent granulomatous eruption in these areas (Figs 1 and 2) and there had been recurrent respira-

tory infections. Over the last 12 months prior to treatment with transfer factor, he was on an antifungal drug, Clotrimazole (Bayer 5097) which partially controlled the infection. When it was attempted, however, to take him off this drug, he immediately relapsed, and the lesions again spread quickly over his face.

Physical examination

The child was of normal intelligence but markedly retarded in growth, height and weight being on the 3rd percentile. There were severe hyperkeratotic granulomata on his face, scalp and the terminal phalanges of many fingers and toes. Teeth had been removed because of chronic infection and the palate was inflamed. The liver and spleen were palpable 2 cm below the costal margins and lymph nodes were slightly enlarged in all areas.

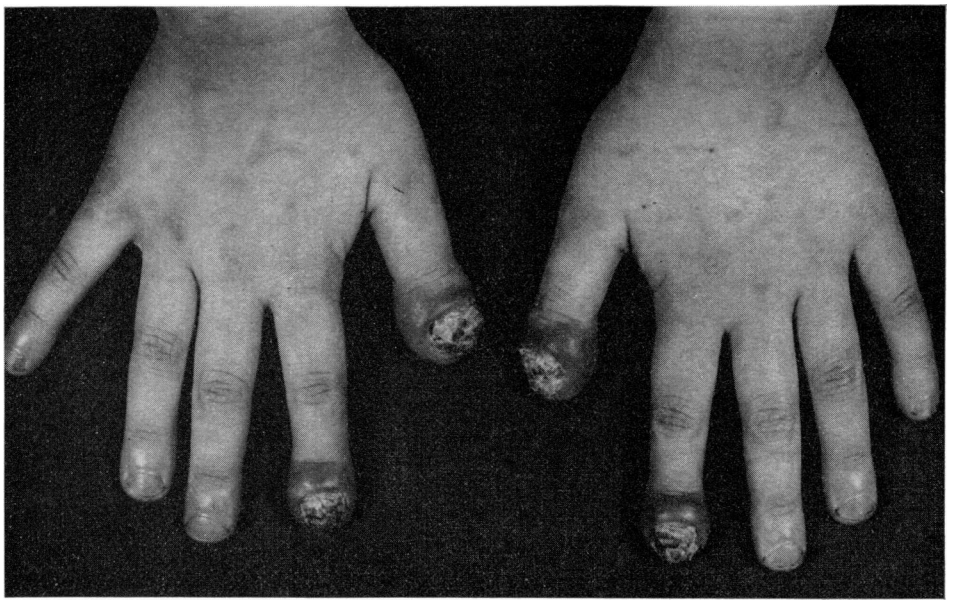

FIG. 2. The patient's hands showing typical features of chronic candidiasis.

Investigations

Hb 9·2–11·6 g%, WBC 4-10000/mm^3, lymphocytes 1400–1700/mm^3. Serum iron 26–87 μg/100 ml, total iron binding capacity 462 μg/100 ml. Blood urea, serum electrolytes, calcium and phosphate normal. Alkaline phosphatase was normal for his age. Serum tyrosin, cortisol and catecholamine were normal. No autoantibodies were detected in the serum. Over the year prior to the injection of transfer factor (TF) serum levels of IgG and IgM were normal for his age. Serum IgA was at times subnormal for his age and ranged from 32–140 mg/100 ml, but a normal IgA level of 9 mg/100 ml was found in his saliva. The patient was of blood group O and iso-haemagglutinins were detected in serum to a dilution of 1/16 for anti-A and 1/4 for anti-B. Antibodies to *C. albicans* were detected by agglutination to a dilution of 1/64, but precipitins to candida extract could not be detected in unconcentrated serum by the Ouchterlony technique. Polymorphonuclear cells (PMN) and monocytes stained for myeloperoxidase (Kaplow, 1965), nitroblue-tetrazolium was normally reduced by these cells

in vitro (Baehner & Nathan, 1968). The ability of his PMN cells to kill *Candida albicans in vitro* was tested by the method of Lehrer & Cline (1970) and he scored 12% on two occasions. (Normal range for adults 14–24%.)

MATERIALS AND METHODS

Materials

Burroughs Wellcome 199 medium was used in all tissue culture work. Phytohaemagglutinin (PHA) Batch BR6 was obtained from Burroughs Wellcome. Concanavalin A (Con A) twice crystallized (lot No. 29) was purchased from Miles Labs. A preparation of candida antigens for the *in vitro* studies was generously donated by Dr Helen Buckley (London School of Hygiene and Tropical Medicine). For intra-dermal injections 1/10 dilution of Bencard candida extract (10% w/v) was used. Purified protein derivative (PPD) batch 282 was supplied by the Weybridge Laboratories. Dinitrochlorobenzene (DNCB) was purchased from British Drug Houses Ltd. ^{3}H-thymidine (specific activity 5-Ci/mmole) was obtained from the Radiochemical Centre, Amersham.

Skin tests

Delayed hypersensitivity (DH) was tested by an intradermal injection of 0·1 ml of the candida extract and 10 units of PPD. The reactions were observed at 12-hourly intervals for 3 days. Induration of 5-mm diameter or more was considered a positive reaction if present 24–72 hr after the intradermal injection. For sensitization 10 mg of DNCB dissolved in 0·1 ml of acetone was allowed to dry on the skin of one forearm. Ten days later a test dose of 50 μg was applied to the skin of the opposite forearm. A positive response was recorded if erythema together with induration appeared 24–72 hr later. Con A 1 μg in 0·1 ml saline, and 0·1 ml of the partially purified migration inhibitory factor (MIF) preparation (see below) was also injected intradermally into each forearm. The injection sites were observed at 6–8-hourly intervals for 36 hr and a positive reaction was recorded if an induration of 5-mm diameter or more developed between 6 and 36 hr after the injection. Skin window tests were done according to the method described by Rebuck & Crowley (1955). Two drops of the test solution were applied under each coverslip and these were removed at 3, 4, 5, 6, 9 and 24 hr. The cells were stained (Wright) and 200 counted on each coverslip, those with less than 200 adherent cells being discarded.

In vitro lymphocyte transformation

The general method has been described in an earlier publication (Valdimarsson *et al.*, 1970). For testing the *in vitro* reactivity of the patients' lymphocytes to candida antigens when cultured in the presence of a dialysable extract from candida sensitive lymphocytes (TF) the following four sets of culture were each made in quadriplates: (1) Lymphocytes only. (2) Lymphocytes and TF. (3) Lymphocytes and candida antigens. (4) Lymphocytes, candida antigens and TF. In set 2 and 4, transfer factor corresponding to a dialysate extracted from 10^6 lymphoid cells was added to each tube. The cultures were terminated at 6 days, 1 μCi of 3H-thymidine having been added to each tube 24 hr previously. The results are expressed as a ratio of the mean radioactivity of antigen stimulated cells over that of the same cells without stimulation. This ratio is called the Index of Replication Activation (IRA). Cytotoxicity assays were done as described earlier (Valdimarsson *et al.*, 1972a).

MIF assay

For assessing MIF activity we have employed an indirect system which is described in detail elsewhere (Valdimarsson *et al.*, 1972a). To exclude inhibition of macrophage migration due to immune complexes (Spitler, Huber & Fudenberg, 1969) all supernatants were filtered through Diaflo × M100 (Amicon) filters.

Preparation of MIF

The method used for preparing the human MIF will be described in more detail in a forthcoming publication (Valdimarsson & Gross, 1972). The blood donors were tested for the presence of Australia antigen in their serum and their lymphocytes, 5×10^6/ml, were incubated in medium 199 without serum. For activation Con A was added to a final concentration of 50 μg/ml of culture medium. Control cells, 5×10^6/ml were left unstimulated. After 24 hr the supernatants were harvested and centrifuged at 3000 rev/min for 15 min. After harvesting Con A was added to the medium from the unstimulated cells. The supernatants were concentrated by ultrafiltration and fractionated on a Sephadex G-200 column. The peaks corresponding to albumin were dialysed against 2000 volumes of pyrogen-free water and freeze dried. The freeze dried material was dissolved in sterile saline, 1 ml for every 10^7 lymphocytes in the original culture. Human erythrocytes were not agglutinated in these solutions. The fractions were finally filtered through millipore membrane (0·22 mμ) tested for sterility and kept as 0·5-ml aliquots in sealed ampoules. It was found that ten-fold dilution of the fraction containing the products of the stimulated cells had a marked inhibitory activity on macrophage migration (42%) whereas the control fraction had no inhibitory activity. Their activity *in vivo* was tested in a blind manner.

Preparation of transfer factor

Potential blood donors were screened for Australia antigen and tested by intradermal injections of 0·1 ml of 1% w/v of the Bencard candida extract. Donors with an induration of 15 mm or more 48 hr after the injection were selected. The blood was collected into a plastic bag (Fenwal) containing acid citrate dextrose. The red cells were sedimented with the aid of dextran, the leucocytes harvested and the number of mononuclear cells determined.

After one quick wash in distilled water the leucocytes were resuspended in a small volume of pyrogen-free water. After five cycles of freezing and thawing and ultrasonication for 40 sec, the resulting cell fragments were dialysed for 48 hr against 200 volumes of distilled water. The dialysate was freeze-dried and redissolved in a small volume of pyrogen-free water. After filtration through millipore membranes (0·22 mμ) and sterility testing, the extract was injected subcutaneously into both shoulder regions.

RESULTS

Before transfer factor was injected the patient developed an immediate response to the candida extract which had completely disappeared 12 hr later. There was no skin response to PPD and two attempts at DNCB sensitization failed. His lymphocytes were normally activated to lymphotoxin production and DNA synthesis in vitro after stimulation with PHA and foreign lymphocytes. Furthermore, following in vitro challenge with soluble candida components they produced MIF activity. This candida activation was not, however, accompanied by an increase of DNA synthesis (Table 1). Only when the patient's lymphocytes

were cultured with candida antigens in the presence of the dialysable transfer factor, did the activation of DNA synthesis reach a significant level (IRA 2·5). His serum did not inhibit the blastoid response of normal lymphocytes to candida antigens.

Because the patient failed to express delayed hypersensitivity in spite of normal production of MIF activity *in vitro*, it was decided to study his skin reactions to Con A and a solution containing MIF activity. Intradermal injection of 1 μg of Con A consistently produced a delayed skin reaction in ten healthy volunteers. We found that this reaction was indurated, reached a maximum within 18–24 hr after the injection and could last for several days. Skin window tests revealed that even as early as 4 hr after the application of Con A to the skin abrasion, the glass adherent cells in the inflammatory exudate were predominantly macrophages, and this cell type dominated the picture throughout the 24-hr test period.

TABLE 1

	Treatment and clinical progress	IRA ± SEM	MIF index ± SEM	Skin reactions		
				Candida	DNCB	MIF
Nov. 1970		0·9 ± 0·2	ND	–	–	ND
Feb. 1971		1·8 ± 0·4	64 ± 7·7	–	–	–
Mar. 1971	Transfer Factor from 9×10^8 cells					
April 1971	Improvement	8·4 ± 1·5	72 ± 7·8	+	–	+
Sept. 1971	Relapsed	1·8 ± 0·1	78 ± 6·2	–	ND	ND
Oct. 1971	Transfer Factor from 8×10^8 cells					
Dec. 1971	Improvement	3·7 ± 0·7	ND	+	ND	ND
* Normal Levels		5·7 ± 3·6	59·8 ± 24·0			

* Means ± 2SD for twenty healthy controls. ND = Not done.

An intradermal injection of the MIF preparation caused a reaction in the skin of healthy individuals as well as in patients with various diseases.* This reaction began after 4–6 hr, was maximally indurated after 16–20 hr and had usually disappeared within 36 hr. When applied under a skin window the mononuclear phase of the cellular response, produced by MIF, was accelerated (Valdimarsson & Gross, 1972).

Neither Con A nor the MIF preparation induced any significant inflammatory response after intradermal injection into the patient. There was only a slight non-indurated erythema which faded within 16 hr. However, in spite of the skin anergy, there was a normal accumulation of glass adherent cells in the patient when candida antigens as well as the MIF preparation were used in the skin window test (Figs 3 and 4). Not only did the mononuclear cells appear at a normal rate, but the total number of glass adherent cells was usually higher on the patient's coverslips than on the control coverslips. Judged by light microscopy the cells were morphologically normal, and their ability to ingest heat killed *C. albicans* applied under the coverslips, compared favourably with control cells.

* These tests were done at the Institute of Diseases of the Chest, London.

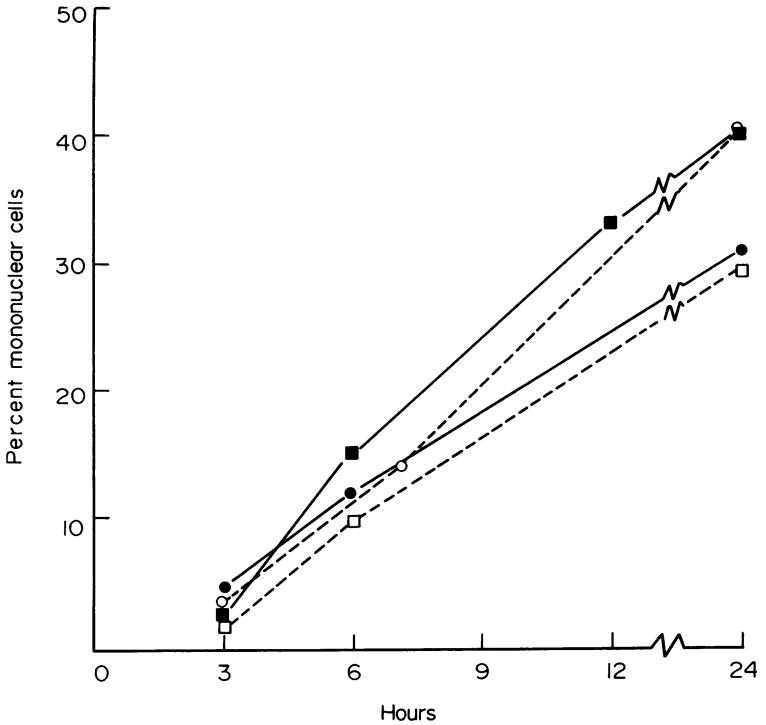

FIG. 3. Rebuck Skin Window test—response to candida antigens. Patient 1 is the patient described in this paper. □, Control 1; ○, control 2; ■, patient 1; ●, patient 2.

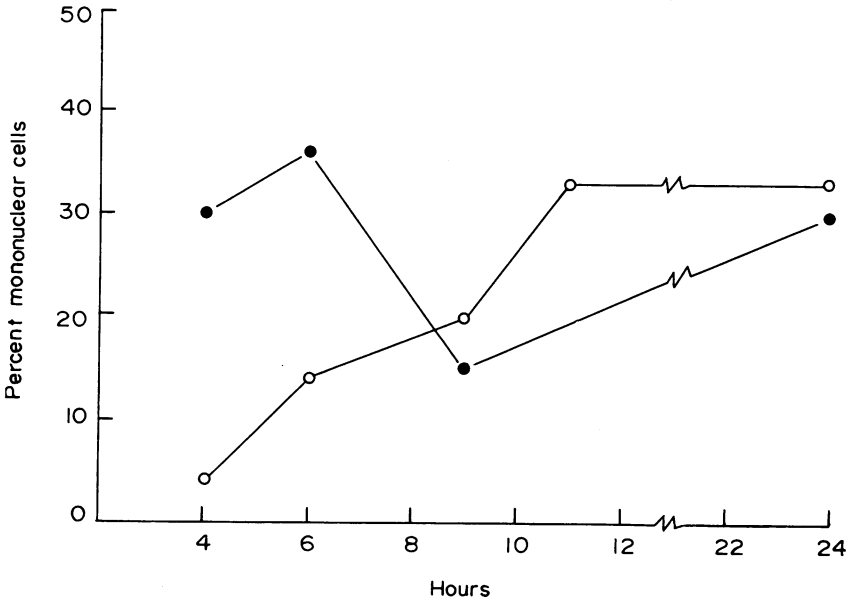

FIG. 4. Rebuck Skin Window Test—response to (○) candida antigens (CA) and partially purified (●) MIF. Compared with the cellular response to candida antigens, the MIF preparation caused a marked acceleration of the mononuclear phase. Similar acceleration was seen in healthy control subjects.

Treatment and progress

As the patient's lymphocytes showed some functional improvement when cultured *in vitro* with the dialysable transfer factor it was decided to attempt to treat him by injection of leucocyte dialysate from donors with strong delayed hypersensitivity to candida. In March 1971 he therefore had subcutaneous injections of an extract derived from 9×10^8 mononuclear cells. Systemic treatment with Clotrimazole was stopped at this stage, but oral iron was given throughout the period. Table 1 summarizes his subsequent clinical and immunological progress. There was an impressive clinical improvement associated with normalization of his immune responses. One month after the first injection the granulomata had almost

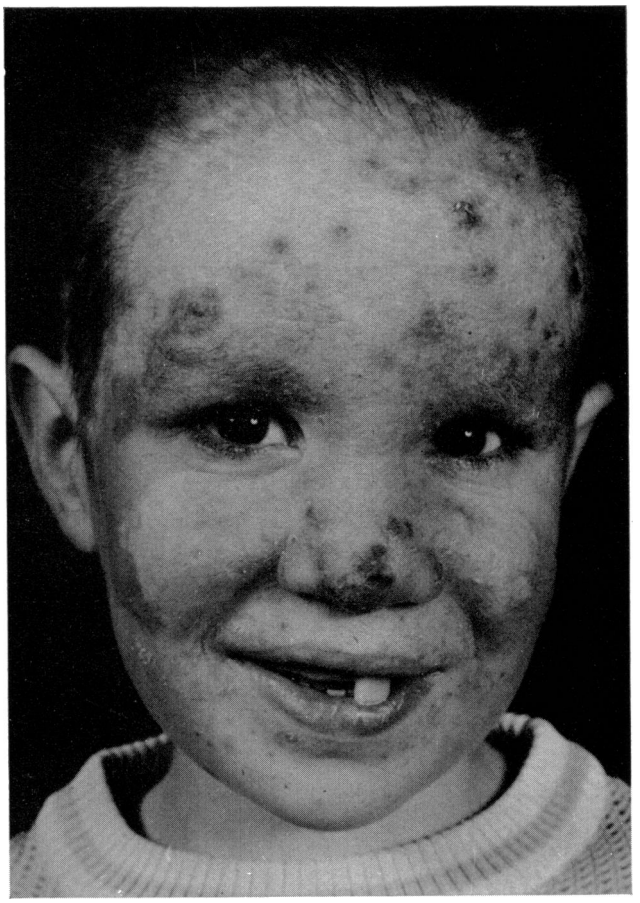

FIG. 5. April, 1971, 1 month after the first injection of transfer factor.

disappeared from his face (Fig. 5) and his general health was better than ever before. Two months later, however, he developed a viral infection, which was thought to be measles and subsequently the fungal infection began to relapse and his general health to deteriorate. In July 1971 systemic treatment with Clotrimazole was started again, but this time the drug failed to control the infection. In spite of continuous systemic and local treatment with

antifungal drugs the granulomata were rapidly recurring when he had the second injection of transfer factor early in October 1971 (Fig. 6). Again there was good clinical and immunological response. As shown on Fig. 7 the lesions on his face had disappeared 2 months later and he started to grow normal nails and the paronychia was also slowly improving. His lungs had improved and his general health was good. Fig. 8 shows his height and weight charts.

In view of the previous experience it was decided to give him the third injection before

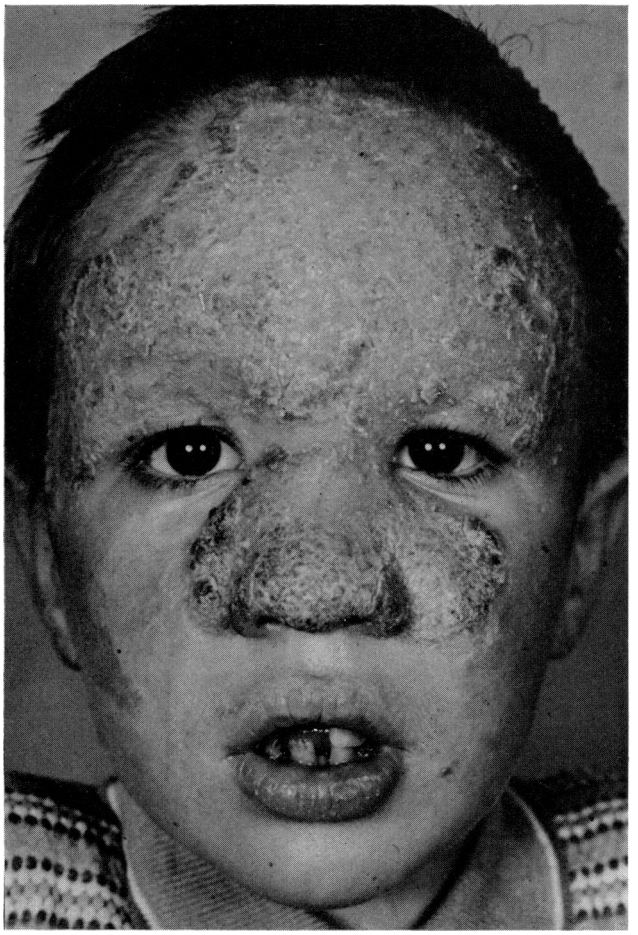

FIG. 6. The patient in October, 1971, before the second injection of transfer factor was given.

he relapsed. The present plan is to inject an extract from 10^9 mononuclear cells every 3–4 months for as long as required.

DISCUSSION

The association of chronic mucocutaneous candidiasis with inability to express delayed hypersensitivity, together with absent lymphocyte transformation but normal MIF produc-

tion *in vitro* after stimulation with candida extract was first demonstrated by Goldberg and his co-workers (1971) and the same pattern has emerged in the patient described here. Interestingly, both these patients were affected by the granulomatous variety of the disease. On the other hand the MIF deficient patients which we have so far investigated have all had macular and scaling dermatitis without skin granulomata. Thus, if it is accepted that MIF acts *in vivo* as a macrophage immobilizer, it is easy to see why patients who are able to produce MIF but whose infiltrating macrophages are unable to eliminate the antigenic

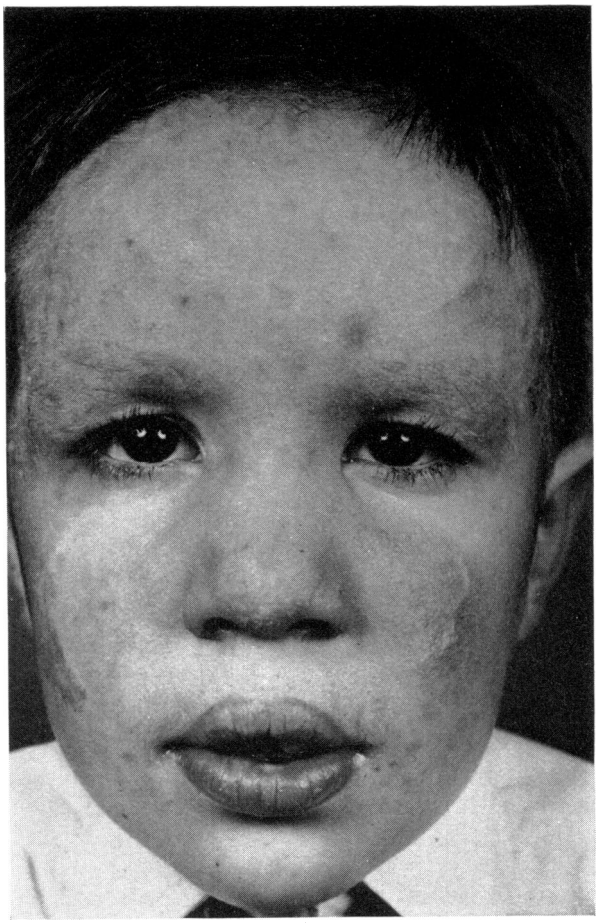

FIG. 7. The patient in December, 1971, 2 months after the second injection of transfer factor.

substance from their tissues, respond by granulomata formation. For the same reason MIF deficient patients would not be expected to respond to their infection by an excessive accumulation of macrophages around the foreign material.

The failure of activation of DNA synthesis in spite of normal release of MIF activity should be emphasized. It could reflect that different classes of lymphocytes are involved in MIF production and the generation of memory cell populations. However, it has been shown that MIF is only released when lymphoid cells pass from the G_1 to the S phase of

their reproductive cycle (Thomas, 1972, personal communication). It is therefore conceivable that lymphocytes might be activated to MIF production without proceeding to DNA replication and mitosis.

The patient was not able to express delayed skin reactions although he was capable of normal MIF production and there was an abundant accumulation of macrophages to the sites, where antigen or MIF was applied.

Three possible explanations may be suggested. Firstly, there could be a lack of some tissue component(s) (not derived from the infiltrating cells) which may be needed for the initiation

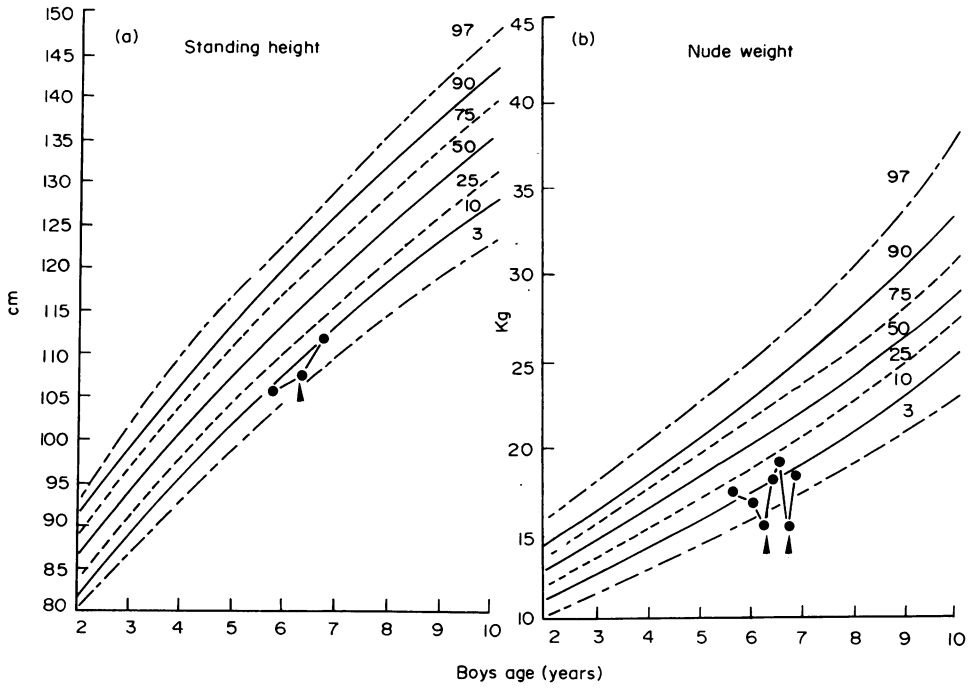

FIG. 8. Height and weight percentile charts. The arrows indicate when transfer factor was injected. (a) Standing height; (b) nude weight.

or full expression of delayed inflammatory reactions (Marmor & Barnett, 1968). Such factors have never been demonstrated, but their existence has not been excluded either.

Secondly, the patient could be unable to produce lymphocyte factors such as macrophage activating factor (David, 1971), or lymphocyte chemotactic factor (Ward, Offen & Montgomery, 1971) which may be distinct from MIF. The observation that an intradermal injection of preformed products of normal lymphocytes did not produce an inflammatory response in the patient, argues against this possibility.

The third possibility is that the patient has a functional defect in his monocyte-macrophage cell line. The inflammation associated with delayed hypersensitivity is possibly largely effected by phlogistic agents which are released from macrophages (Hill, 1969). There is also circumstantial evidence for the existence of mutually stimulating interaction between lymphocytes and macrophages *in vivo* (Unanue, Askonas, & Allison, 1969; Humphrey, 1970). Thus, if monocytes having been immobilised and activated by an adequate lymphocyte

function, do not produce or release such component(s), expression of DH reactions might fail to occur. This, we believe, could be the basic defect of some patients with granulomatous candidiasis. It should be pointed out in this connection that in our patient the lymphocytes responded normally *in vitro* to PHA which does not require the presence of macrophages. Furthermore, although unable to transform in response to soluble antigens, which is probably a macrophage dependent reaction, they were normally activated to blastoid transformation in the mixed lymphocyte culture system where competent macrophages are provided by Mitomycin treated foreign cells.

In view of the postulated macrophage defect in our patient, his response to the leucocyte extract was surprising, and we are unable to offer any suggestions as to the mechanisms responsible for this effect. It is, of course, possible, although unlikely, that the clinical improvement was not caused by TF but resulted from another factor present in the leucocyte dialysate. The chemical nature of TF remains to be elucidated, however, and our findings might therefore also suggest that current ideas about the mechanism of TF action might be wrong.

The infection in our patient had already been partially contained by an antifungal drug when TF was given. However, the acute relapse which followed an attempt to stop the drug prior to the TF injections together with the fact that the patient continued to improve for 2 months after the initial injection without any systemic drug treatment, certainly suggests that the dialysate was effective. Furthermore, when he finally relapsed three months after the first injection, he was again given the antifungal drug, which this time did not prevent his infection from progressing. An injection of TF was, however, again followed by a dramatic improvement. We are, therefore, encouraged to continue this treatment and explore its potential in other cases of chronic infections which fail to respond to conventional treatment.

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ABBREVIATIONS

DH	delayed hypersensitivity
TF	transfer factor
Con A	concanavalin A
IRA	index of replication activation