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PRECIPITINS TO *CANDIDA ALBICANS* IN CHRONIC MUCOCUTANEOUS CANDIDIASIS STUDIED BY CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL

CORRELATION WITH CLINICAL AND IMMUNOLOGICAL FINDINGS

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SUMMARY

The precipitating antibodies in the sera of fifteen patients with chronic mucocutaneous candidiasis were examined by crossed immunoelectrophoresis with intermediate gel. The method permitted identification and quantitation of precipitins against thirty-four of the seventy-eight known antigenic components of *Candida albicans*.

The sera from every patient contained precipitins and the number of reactivities per serum ranged from two to thirty-nine. All patients had antibodies to antigen 78, a mannan–protein complex. Many sera also possessed antibodies to many other components of the organism, suggesting that some of the yeast cells had been disrupted in the patients' tissues. However, there were no precipitin profiles that characterized patients with specific forms of chronic candidiasis. Instead, in two cases, the antibody profiles appeared to be related to the patients' ability to develop humoral immune responses. Serial studies of patients during remissions and exacerbations showed that there were no consistent changes in antibody activities.

The role of *Candida* precipitins in chronic candidiasis remains uncertain. Possible functions include prevention of dissemination of the infection from superficial sites, formation of immune complexes in superficial sites and suppression of cell-mediated immunity as suggested by *in vitro* tests.

INTRODUCTION

Substantial evidence has accumulated indicating that a basic predisposing factor in chronic mucocutaneous candidiasis (CMC) is deficiency of the cellular immune apparatus. Therefore in recent years only little attention has been paid to serum antibodies against *C. albicans* in CMC, and in most studies the finding of *Candida* agglutinins and/or precipitins has been

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used merely to support the view that the humoral immune apparatus is not deficient in most of these patients (Kirkpatrick, Rich & Bennett, 1971). However, several observations in CMC leave room for the possibility that humoral immunity may also be involved in this disorder, e.g. the unexplained intense dermal inflammation (Kirkpatrick *et al.*, 1971; Lehner, 1966), the frequent occurrence of antibodies to endocrine tissues (Louria *et al.*, 1967; Blizzard & Gibbs, 1968) and the fact that restoration of cellular immune competence has not always provided complete clinical remissions (Levin, Spilter & Fudenberg, 1973; Kirkpatrick & Smith, 1974).

Due to recent developments in quantitative immunoelectrophoresis (Laurell, 1972; Axelsen, Krøll & Weeke, 1973) seventy-eight antigens have been characterized in *C. albicans* (Axelsen, 1973a) and in one CMC-serum twenty-two *Candida* precipitins were found (Axelsen, 1971; Svendsen & Axelsen, 1972), and in still another CMC-serum simultaneous occurrence of *Candida* precipitins and antigens were demonstrated (Axelsen & Kirkpatrick, 1973). These observations suggested that a more intensive study of *Candida* antibodies in CMC might provide additional insight into the pathogenesis of this syndrome. In this report the results of the analysis of thirty-three sera from fifteen CMC patients are summarized. The aim was to identify and titrate the *Candida* precipitins in terms of the seventy-eight antigen schemes recently described (Axelsen, 1973a), and to correlate the findings with the clinical and immunological features of the patients.

MATERIALS AND METHODS

Patients and sera

The clinical and immunological features of the fifteen CMC patients are summarized in Tables 1 and 2. Ten patients were hospitalized at the Clinical Center at NIH, and five (M.R., S.H., R.B., K.F., M.H.) at Duke University Medical Center. Certain clinical and immunological features of some of these patients have been published elsewhere: R.H., G.R., K.B., J.C., J.B., S.M., D.L., H.S. (Kirkpatrick *et al.*, 1971) and M.R. (Buckley *et al.*, 1968). Five patients (R.H., K.B., J.C., J.B. and M.R.) were followed through remissions and relapses. In each case, the infecting organism was identified as *Candida albicans*, and no other *Candida* species were isolated.

The extent of candidiasis was scored according to the following scale: none = no lesions; minimal = residual lesions that are regressing during or following therapy; 1+ = involvement of one mucous or dermal site; 2+ = involvement of one dermal + one mucous membrane site, or two dermal sites; 3+ = involvement of more than two dermal sites + mucous membranes; 4+ = generalized.

Impaired humoral antibody responsiveness to a panel of test antigens was demonstrated in two of the patients, K.F. and R.B. (Buckley, Wray & Belmaker, 1972; Buckley, unpublished data), despite low normal or normal concentrations of serum immunoglobulins.

Disorders of endocrine function were present in five patients: one (R.H.) had hypoparathyroidism; one (R.V.) had juvenile-onset diabetes mellitus; three patients (J.C., J.B. and G.R.) had hypothyroidism. One patient (G.R.) also had adrenal insufficiency.

Sera were stored at −40°C for up to 5 years before analysis. Prior to mailing to Copenhagen NaN_3 was added to a final concentration of 15 mM.

TABLE 1. Clinical features of fifteen patients with chronic mucocutaneous candidiasis

Patient	Age/Sex	Years of candidiasis	Affected sites						
			Mouth	Vagina	Nails	Skin			
						Hands	Feet	Scalp	Other
R.H.	19/M	18	+		+	+	+	+	Generalized
G.R.	24/M	22	+		+				
K.B.	18/F	6	+	+	+	+	+	+	Generalized
J.C.	24/F	24	+	+	+	+	+		Axilla
J.B.	24/F	15	+	+	+	+			
S.M.	27/F	23	+	+	+				
C.B.	17/M	9	+		+	+	+		
R.V.	33/F	30	+	+	+	+	+	+	
D.L.	23/M	22	+		+			+	
H.S.	10/M	4	+		+	+			
M.R.	10/F	9	+	+	+	+		+	Generalized
S.H.	10/M	10	+		+	+	+		
R.B.	14/M	14	+		+	+		+	
K.F.	7/M	3	+		+				Shoulder
M.H.	5/M	4	+		+	+			Perineum

TABLE 2. Cellular immune responses in fifteen patients with chronic mucocutaneous candidiasis

Patient	Delayed skin responses		Lymphocyte transformation		MIF production	
	Candida	Other antigens	*Candida*	Other antigens	*Candida*	Other antigens
R.H.	Negative	Negative	Positive	Positive	Negative	Negative
G.R.	Positive	Negative	Positive	Negative	n.d.	n.d.
K.B.	Negative	Positive	Positive	Positive	Negative	Positive
J.C.	Negative	Negative	Negative	Negative	Negative	Negative
J.B.	Positive	Positive	Positive	Positive	n.d.	n.d.
S.M.	Negative	Positive	Negative	Positive	Negative	Positive
C.B.	Negative	Positive	Negative	Positive	Negative	Positive
R.V.	Negative	Negative	Negative	Negative	Negative	Negative
D.L.	Negative	Negative	Negative	Negative	Negative	Negative
H.S.	Positive	Positive	Positive	Positive	Negative	Positive
M.R.	Negative	Negative	Negative	Positive	n.d.	n.d.
S.H.	Positive	Positive	Positive	Positive	Positive	Positive
R.B.	Negative	Negative	Positive	Positive	Positive	Positive
K.F.	Positive	Positive	n.d.	n.d.	n.d.	n.d.
M.H.	Positive	Positive	Positive	Positive	n.d.	n.d.

N.d. = not determined.

Crossed immunoelectrophoresis with intermediate gel

The technical and theoretical details of this method have recently been reviewed (Axelsen, 1973b). The electrophoreses were run using the DL immunoelectrophoresis equipment (Dansk Laboratorieudstyr Ltd, Copenhagen, Denmark) and 1% (w/v) agarose gel (Litex,

Glostrup, Denmark) in barbital buffer, pH 8·6, ionic strength 0·02. The temperature of the cooling water was 12°C. The thickness of gels was 1 mm.

The batch of *Candida albicans* antigen, *St. Ag.* 71, and the batch of rabbit IgG anti-*C. albicans*, *St. Ab.* 3–8, were the same as described in detail earlier (Axelsen, 1973a).

Two microlitres of antigen (*St.Ag.* 71 diluted with 0·15 M NaCl, 1:1) were submitted to first dimensional electrophoresis, applying 10 V cm^{-1} for 55 min. The second dimensional electrophoresis was run overnight applying 2 V cm^{-1}.

The intermediate gel contained patient's serum in the test plates (13·6 μl of serum/cm^2 of gel), or saline in the control plates (the volume of 0·15 M NaCl was equal to that of serum in the test plates). The reference gel contained *St. Ab.* 3–8 (5 μl/cm^2 of gel). The dimensions of the plates were 10 × 10 cm; of the intermediate gel 2 × 10 cm; and of the reference gel 6 × 10 cm. After the electrophoreses the plates were washed in 0·1 M NaCl (overnight), distilled water (1 hr), pressed under filter paper and soft blotting paper (20 min), dried under a hair drier, and finally stained (5 min) in Coomassie Brilliant Blue R (Michrome, Edward Gurr Ltd, London; 5% (w/v) in ethanol–glacial acetic acid–water (45:10:45)) and destained (5 min × 3) in the solvent.

Examination of the precipitate patterns was performed by looking for differences between the test plates and the corresponding control plate; the differences were interpreted as described by Axelsen (1973b). In the procedure used in this study seven test plates and one control plate were run per day; the eight first dimensional electrophoreses were run simultaneously in the same apparatus. In the control plates about fifty precipitates were seen; in the test plates the following thirty were scrutinized routinely: 7, 8, 11, 13, 15, 16, 18, 21, 25, 28, 29, 33, 40, 44, 46, 47, 49, 51, 56, 57, 58, 59, 61, 62, 63, 64, 65, 71, 76 and 78 (the numbers refer to the antigen scheme given elsewhere (Axelsen, 1973a). In some patients' sera precipitins had specificities for other antigens than the above-mentioned, and some could not be identified. The identifiable human precipitins were titrated (semiquantitatively) by comparing each precipitate of the test plates with the corresponding precipitates in plates containing *St. Ab.* 3–8 in the intermediate gel. The titres of the human antibodies were expressed in arbitrary units which were derived from experiments with various concentrations of *St. Ab.* 3–8 in the intermediate gel. According to this: titre 8 $\geqq$ 405% > titre 7 $\geqq$ 270% > titre 6 $\geqq$ 180% > titre 5 $\geqq$ 135% > titre 4 $\geqq$ 90% > titre 3 $\geqq$ 45% > titre 2 $\geqq$ 9% > titre 1 > titre 0 = 0%. The percentages signify the concentration of the *St. Ab.* 3–8 in the intermediate gel in relation to the concentration of the reference gel (100% = 5 μl of *St. Ab.* 3–8/cm^2 of gel). This scoring system is illustrated in Fig. 2. On the background of studies on precision (day to day variation) a titre difference between two sera should be two titre units to be ascribed significance. The precipitin score of each serum was calculated as follows: 1 point for an antibody with titre 1, $1\frac{1}{2}$ points for titre 2, 2 points for titre 3 *etc.*, and 1 point for an unidentified antibody.

RESULTS

Fig. 1 shows two crossed immunoelectrophoreses with intermediate gel, a control plate (a), and a test plate (b) containing the serum from patient R.H. (July, 1969). This specimen contained thirty-nine precipitating antibodies, thirty of which could be identified in terms of the reference nomenclature of Axelsen (1973a). This *Candida* precipitin response is the strongest found in man thus far and is equal to that found in many rabbits immunized

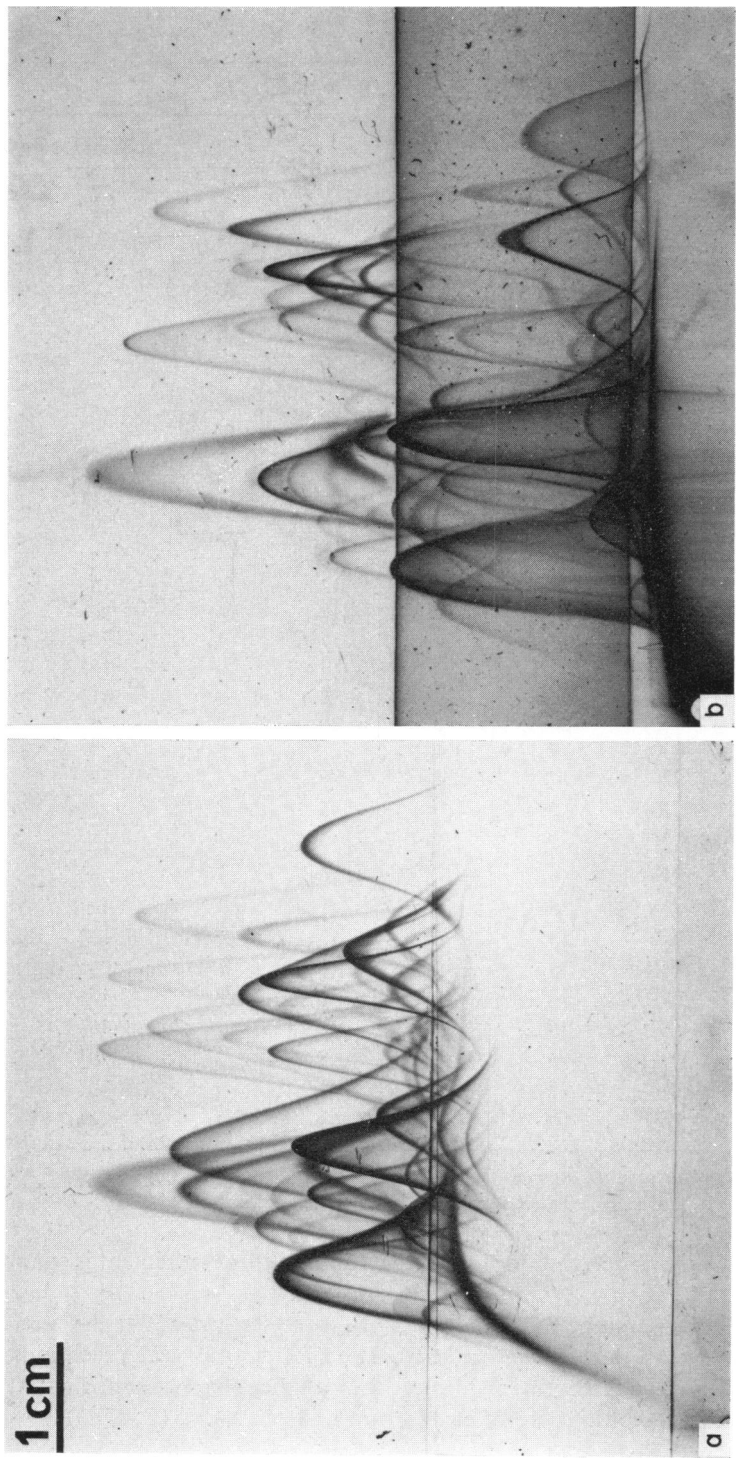

FIG. 1. Crossed immunoelectrophoresis with intermediate gel. (a) Control plate with saline in the intermediate gel. (b) Test plate with serum from a patient (R.H., July 1969) in the intermediate gel. This precipitin response to antigens of *Candida albicans* is thus far the strongest found in man. The detailed interpretation into precipitin specificities and titres appears from Fig. 2. Technical points: the anode was to the right during first dimension electrophoresis and the anode was at the top during second dimension electrophoresis; staining was with Coomassie Brilliant Blue R.

TABLE 3. Precipitins in thirty-three sera from fifteen patients with chronic mucocutaneous candidiasis

Patient	Date of sampling	Precipitins				
		Identified	Unidentified	Total	Highest titre	Score†
R.H.	(13/6/69)*	13	3	16	8	27
	(31/7/69)	30	9	39	8	75
	(11/2/70)	27	3	30	8	66
	(25/6/70)	29	7	36	8	74½
	(5/10/70)	29	7	36	8	73
	(14/1/71)	30	6	36	8	73
	(27/4/71)	30	8	38	8	72
	(29/6/71)	28	11	39	8	72
G.R.	(16/3/70)	6	0	6	2	6½
K.B.	(24/6/70)	15	4	19	8	37½
	(11/8/70)	15	4	19	8	38
	(7/4/71)	14	4	18	8	31½
	(28/6/71)	13	4	17	8	29
J.C.	(25/5/70)	12	0	12	6	17
	(28/9/70)	12	0	12	4	16½
	(8/1/71)	12	0	12	4	16½
	(27/4/71)	15	0	15	4	20½
	(29/6/71)	14	0	14	4	18½
J.B.	(24/2/70)	21	1	22	8	41½
	(13/4/70)	20	0	20	8	38
	(28/4/70)	20	1	21	8	39
	(12/5/70)	19	1	20	8	38½
S.M.	(24/2/70)	5	0	5	3	7½
C.B.	(19/3/71)	9	1	10	5	15
R.V.	(8/7/71)	6	2	8	2	8½
D.L.	(7/7/71)	14	0	14	5	19
H.S.	(20/8/70)	13	0	13	5	21½
M.R.	(15/8/66)	18	0	18	6	29
	(8/4/68)	12	0	12	3	15
S.H.	(1/11/69)	10	0	10	6	18½
R.B.	(27/1/71)	6	1	7	3	9½
K.F.	(10/12/68)	2	0	2	1	2
M.H.	(9/2/70)	3	0	3	3	4½

* In this serum antigens were also found.
† Normal range: 0–6½ (Axelsen & Andersen, to be published).

against *C. albicans* (Axelsen & Svendsen, 1972). The result of this experiment is summarized in Fig. 2.

The total number of precipitins found in the thirty-three sera was 599; the number per serum ranging from two to thirty-nine (Table 3). The specificity of 522 precipitins (87%) could be determined and were confined to thirty-four of the reference antigens. After the titre determination the precipitin score of each serum was calculated (Table 3).

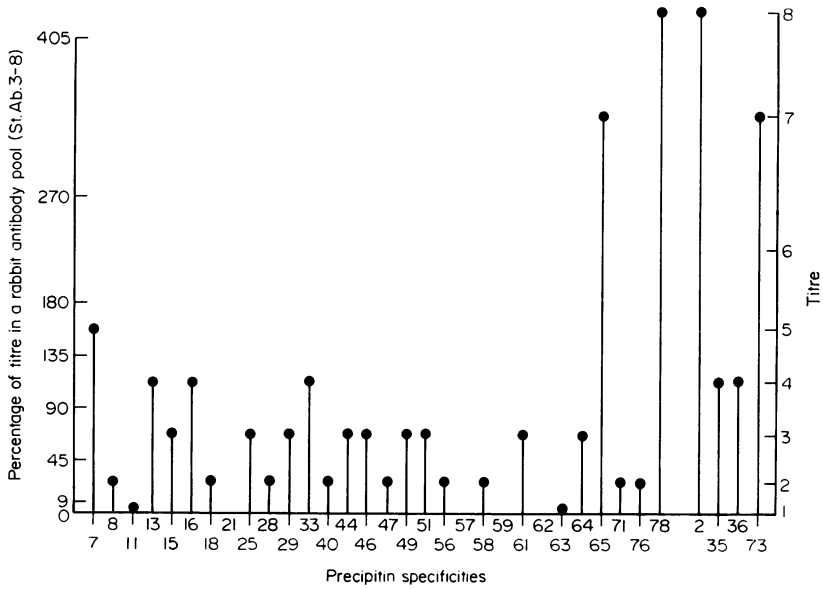

FIG. 2. Evaluation of the precipitin findings of Fig. 1. The precipitin specificities are in accordance with the *C. albicans* antigen scheme described elsewhere (Axelsen, 1973a).

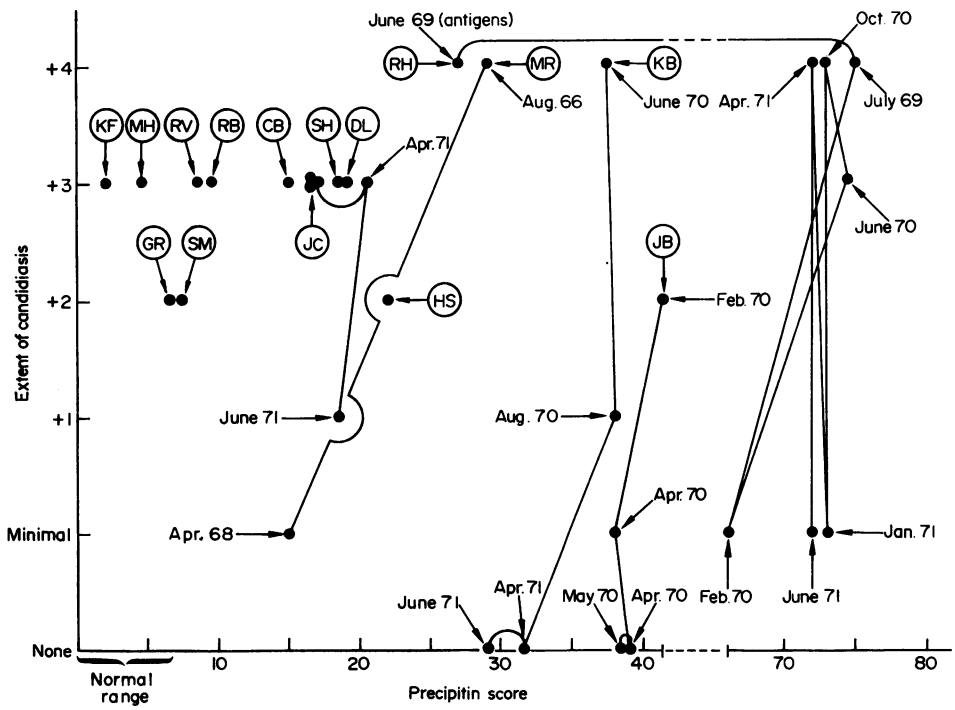

FIG. 3. Lack of correlation between the extent of candidiasis and the precipitin score in CMC. Five patients (R.H., J.B., K.B., J.C. and M.R.) were followed during remissions (after therapy with antibiotics or immunorestoration) and relapses.

For five patients serial samples were available; the relationships of the antibody score to the extent of the clinical infection is shown in Fig. 3.

There was no correlation between the duration of candidiasis and the precipitin score. The sera with the largest number of precipitins also had the highest titres (Table 3) and sera with a small number of precipitins had low titres.

In one serum (R.H., June, 1969) six 'free' antigens were found, and of these, five could be identified as antigen numbers 5, 11, 15, 18 and 40. The technical aspects of this finding have been reported in detail elsewhere (Axelsen & Kirkpatrick, 1973).

DISCUSSION

Using the highly resolving and sensitive technique of crossed immunoelectrophoresis with patients' sera in the intermediate gel it has been possible to characterize the specificities and titres of precipitating antibodies to *C. albicans* in patients with CMC. Precipitins were detected in all sera and although the number of precipitins extended from two to thirty-nine, the mean number was 12·7 per patient. All patients had precipitins against antigen 78, a mannan–protein complex. Other than this the reactivities were directed against a variety of *Candida* antigens, suggesting that in most patients intracellular *Candida* antigens had been exposed to the immune apparatus. The individuality of the antibody profile in each patient (data not presented here except for Fig. 2) is in all probability due to differences in the patients' immune responses, since variation in antigen content between strains has been shown to be relatively small (Axelsen, 1973a). Also in favour of this interpretation is the finding that the precipitin responses of individual rabbits, immunized identically with the same complex *Candida* antigen, differ considerably (Axelsen & Svendsen, 1972). The finding that antibody profiles in individual patients were not clearly related to the extent of candidiasis also suggests the contribution of the individuals' immune response. In favour of the latter is the fact that two of the patients (R.B., K.F.) with low precipitin scores were shown to have impaired humoral immune responses to soluble antigens other than *Candida*. A patient, M.H., with a low precipitin score was not evaluated from the standpoint of humoral immune responsiveness. There were no antibody profiles or precipitin scores that were unique to patients with endocrinopathies or serum 'autoantibodies' against endocrine tissues.

The lack of correlation between the precipitin score and the number of endocrine organ-specific autoantibodies (Table 4) is against a thesis that the same immune defect leads to both increased autoantibody formation and increased anti-*Candida* antibody formation. The lack of correlation between negative DCH and autoantibodies (Table 4) is against the suggestion of Provost *et al.* (1973) that the autoantibodies in patients with chronic mucocutaneous candidiasis may be the result of defective modulation of the antibody response by the thymus-dependent immune system.

In five patients it was possible to perform serial measurements of antibody activities during remissions and relapses (Fig. 3). Again, even though therapy produced marked changes in the extent of candidiasis, corresponding significant changes in precipitin scores were observed in only one patient (M.R.). This lack of correspondence was somewhat surprising but suggests again that, rather than the antigenic burden, the individuality of the immune system in these patients is the major decisive factor behind the differences in the precipitin responses.

Patient R.H. presented a unique situation. In June 1969, when he had generalized

TABLE 4. Correlation between the extent of candidiasis, delayed hypersensitivity to *Candida* antigens (DCH), precipitin score and autoantibodies in fifteen CMC patients

Patient	Extent of candidiasis	DCH	Precipitin score	Autoantibodies
R.H.	4+	−	75	Anti-thyroglobulin
G.R.	2+	+	6½	Anti-adrenal; anti-thyroid; anti-thyroglobulin
K.B.	4+	−	37½	Rheumatoid factor
J.C.	3+	−	17	None
J.B.	2+	+	41½	Anti-thyroid; rheumatoid factor
S.M.	2+	−	7½	None
C.B.	3+	−	15	None
R.V.	3+	−	8½	n.d.
D.L.	3+	−	19	None
H.S.	2+	+	21½	None
M.R.	4+	−	29	None
S.H.	3+	+	18½	Rheumatoid factor
R.B.	3+	−	9½	Negative
K.F.	3+	+	2	Negative
M.H.	3+	+	4½	Anti-thyroid; rheumatoid factor

N.d. = not determined.

candidiasis, his serum contained both *Candida* antigens and precipitins (Axelsen & Kirkpatrick, 1973). However, 1 month later, the antigens were no longer detectable and the precipitin score had risen to over 70. Thereafter, the precipitin score remained high. Surprisingly, he had no signs, symptoms or laboratory findings suggestive of diffuse immune complex disease.

It is possible to postulate different roles for antibodies in CMC. On one hand, antibodies may be protective by preventing dissemination of the infection from skin and mucous membranes to parenchymal organs. On the other hand, the antibody molecules may interact with the *Candida* antigens in epidermal and dermal sites with the formation of immune complexes, which in turn activate the complement cascade. This in turn could evoke the local inflammatory responses. In this regard the antigen–antibody interactions would occur in superficial sites and the potential for diffuse immune complex diseases would be minimal. The finding of normal levels of total haemolytic complement in patients with CMC (Kirkpatrick *et al.*, 1971) may be evidence against this proposition. However, more definitive assays such as titration of individual complement components, evaluation of the function of the alternate complement pathway, or serial measurements of complement function during remissions and exacerbations have not been done. Immunopathological studies of the cutaneous lesions in patients with CMC may also clarify this question. Finally, it is also possible that antibodies or antigen–antibody complexes could be the plasma factors which have been shown to inhibit *in vitro* lymphocyte responses in some patients with CMC (Canales *et al.*, 1969; Paterson *et al.*, 1971; Valdimarsson *et al*, 1973).

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