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Intralesional plasma cells and serological responses in human cutaneous leishmaniasis

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SUMMARY

Intralesional plasma cells and serological responses were investigated in 20 Brazilian cases of cutaneous leishmaniasis. Plasma cell numbers varied from less than 10% to more than 50% of cells in inflammatory infiltrates, in general with greater numbers of such cells present in lesions of longer duration. Direct fluorescence examination with anti-IgG, -IgA and -IgM sera of trypsin-treated sections of formalin-fixed biopsy tissue revealed that most intralesional plasma cells contained IgG. Russell bodies were detected in eight cases, in seven of which these bodies fluoresced only with anti-IgM serum. There was no correlation between serum levels of total IgG, IgA and IgM (detected by radial immunodiffusion) or antileishmanial antibodies (detected by class-specific indirect immunofluorescence and by direct agglutination with and without 2-mercaptoethanol) and numbers of intralesional plasma cells of the same globulin class. No striking or consistent alterations in complement components were noted in the serum of these patients.

INTRODUCTION

Cutaneous leishmaniasis is a disease characterized by chronic nodular or ulcerative skin lesions. Cellular immune mechanisms have been considered of primary importance in the eventual cure of these lesions since the development of delayed hypersensitivity to leishmanial antigens (the Montenegro reaction) usually coincides with the first signs of healing of lesions and since positive delayed skin reactions are absent in the severe diffuse form of the disease (Preston & Dumonde, 1976a). Low levels of circulating specific antibodies and the lack of success of most serum transfer experiments have led to the conclusion that humoral immunity plays a minimal role in protection.

However, evidence from experimental models suggests that the contribution of humoral factors in cutaneous leishmaniasis should be re-evaluated. Preston & Dumonde (1976b) transferred lymphoid cells, serum, or both from *Leishmania tropica*-immune mice to normal recipients and showed that greater protection was conferred by simultaneous transfer of both than was obtained by either factor alone. Poulter (1980), studying *L. enriettii* infection in guinea-pigs, noted that serum alone could confer protection if taken from cured animals 24 hr after promastigote challenge.

Plasma cells have long been known to be a striking histological feature of many human cutaneous leishmaniasis lesions (Pessoa & Barretto, 1944) but the significance of this finding has not been fully investigated, and the class of immunoglobulin produced by these cells has not been identified.

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The purpose of the present study was to identify, by immunofluorescence, the class of globulins produced by intralesional plasma cells in human cutaneous leishmaniasis lesions and to examine possible relations between the presence of these cells, the titres of circulating antileishmanial antibodies and alterations of serum proteins. The use of recently developed techniques of trypsinization of paraffin-embedded tissues to improve immunofluorescence sensitivity permitted immunofluorescence and histopathological analysis of adjacent sections of relatively large biopsy specimens.

MATERIALS AND METHODS

Patients. Patients were examined as they became available through local out-patient services. Criteria for diagnosis of cutaneous leishmaniasis included history of exposure, consistent clinical findings including positive Montenegro test and positive serology with leishmanial antigens; in most cases, diagnosis was confirmed by identification of Leishmania in histological preparations or isolation of the organism in culture (Table 1). Serum was collected from each patient and stored at −20°C until tested; plasma was collected with 20 mM EDTA anticoagulant and stored in liquid nitrogen. Punch or elliptical biopsies were taken at the time of blood collection; one tissue fragment was removed aseptically for culture in enriched NNN medium, and another fragment was fixed in buffered 10% formalin, pH 7·2, for histological processing.

Histological procedures. After routine paraffin embedding, adjacent 5-μm sections were stained with haematoxylin & eosin (H&E) or were mounted with LePage Bond Fast glue (LePage Ltd, Montreal, Canada) for immunofluorescence.

Immunofluorescence of tissue sections was carried out after trypsinization (Huang, Minassian & More, 1976; Qualman & Keren, 1979). Deparaffinized sections were digested with 0·1% trypsin (DIFCO 1:250) in 0·1% $CaCl_2$, pH 7·8, washed in distilled water and held overnight in phosphate-buffered saline, pH 7·2 (PBS). After preincubation for 10 min with normal rabbit serum 1:20 followed by a PBS wash, slides were incubated for 30 min at room temperature with one of the following FITC-conjugated antisera at appropriate dilutions: goat anti-human IgM, mu-chain-specific (Hyland Laboratories, California); rabbit anti-human IgG, gamma-chain-specific (Behring Institute, Brazil); goat anti-human IgA (Miles Yeda, Israel). After mounting in buffered glycerol, pH 8·0, slides were observed with a Zeiss microscope with HB200 lamp, incident illumination, B22 exciter filter and G247 barrier filter. Photographs were made on Kodak Plus X or Tri X film.

In this investigation, attention was limited to dermal tissue, which in all cases contained moderate to intense mixed infiltrates of cells of the mononuclear phagocytic system, lymphocytes, plasma cells and occasionally varied numbers of granulocytes. Epidermis and areas of necrosis were not scored. The number of plasma cells in sections was estimated independently by two observers for H&E sections and immunofluorescent sections using the following scoring system: + = plasma cells less than 10%; + + = 10–30%; + + + = 30–50%; + + + + = more than 50% infiltrating cells.

Serology. All antigens were prepared from cultured promastigotes of a previously described strain of *Leishmania mexicana* (Grimaldi, Moriearty & Hoff, 1980). Indirect immunofluorescence was done with organisms acetone-fixed on slides using the FITC conjugates described above. The highest doubling serum dilution giving uniform fluorescence was considered as the titre.

Trypsinized, formalin-fixed promastigotes were used for the direct agglutination test (DAT) (Vattuone & Yanovsky, 1971), carried out in microtitration plates. Serum was tested with and without preincubation for 1 hr with 0·1 M 2-mercaptoethanol (Schmunis, 1978). Reactions at a dilution of 1:160 or higher were considered specific, the titre being the last dilution giving a mat greater than half the well diameter.

Countercurrent immunoelectrophoresis (CCIEF) was performed in 1·5% agarose with barbital buffer, pH 8·2. The antigen was a crude soluble promastigote fraction containing 5 mg protein/ml as determined by the Lowry method with a BSA standard.

For all serological procedures, pooled normal human serum served as a negative control; positive controls included both positive human sera and serum from rabbits hyperimmunized with promastigote antigen.

Serum and plasma proteins. Circulating immune complexes were analysed by the ^{125}I-C1q-binding test (C1qBA) (Zubler *et al.*, 1976). Total haemolytic complement was measured by the CH50 test (Mayer, 1961) using sheep red cells (SRBC) and commercial anti-SRBC serum (Wellcome Reagents Ltd, England), while levels of complement fractions C3, C4 and B were determined by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) using commercial specific antisera (Behring Institute, Brazil).

Results in these tests were compared with those obtained with sera from Swiss blood donors and from normal Rio de Janeiro residents. Total serum levels of IgM, IgG and IgA were measured using commercial radial immunodiffusion plates (Tripartigen, Behring Institute, Brazil).

RESULTS

Intralesional plasma cells

Plasma cells were found in H&E sections of all 20 biopsies examined, ranging in frequency from less than 10% to more than 50% of infiltrating cells (Table 1, Fig. 1). In four cases, Russell bodies were also detected (Fig. 1). Though there was no strict correlation, there was a tendency for plasma cells to be more frequent in lesions of longer duration.

Table 1. Intralesional plasma cell frequency and serological responses of patients with American cutaneous leishmaniasis

Patient data*				Intralesional plasma cell frequency†			Serology‡				
					Fluorescence		IFA titre		DAT	Total globulins (mg%)	
No.	Form	Duration	Diag. crit.	H&E	IgG	IgM	IgG	IgM	titre	IgG	IgM
1	Cut.	6 mo	I	++++	++++	–	40	20	320	2,420	345
2	Cut.	1 mo	I	+++	++ to ++++	+	320	40	80	1,680	135
3	Cut.	8 mo	I	+++, RB	++ to ++++	+, RB	160	—	40	2,640	110
4	Cut.	3 mo	I	+++, RB	+++	+, RB	40	—	20	1,330	90
5	MC	1 yr	I	++	+ to +++	+	1,280	—	640	1,490	211
	Cut.	6 mo	I	+++	++	++, RB					
6	Cut.	2 mo	I	+++	++	+, RB	20	—	20	800	160
7	2 cut.	6 mo	HCS	+++	+++ to ++++,RB	+	320	40	320	1,560	190
8	Cut.	8 mo	HCS	++	+++	+	—	40	80	2,040	92
9	4 cut.	1 mo	I	++	+++	+	—	—	20	1,560	228
10	Cut.	1 mo	I	++	++	+,RB	—	—	20	1,640	155
11	3 cut.	1 mo	I	++	+ to ++	+, RB	40	20	80	2,260	197
12	Cut.	2 mo	HCS	++, RB	++	+	160	—	40	1,600	107
13	n.a.	n.a.	HE	++	+++	+	320	80	160	1,800	n.d.
14	Cut.	6 mo	HE	++	+ to ++	+	20	—	—	1,510	96
15	MC	3 yr	HCS	++, RB	+	+	80	—	20	2,260	230
16	6 cut.	1 mo	I	++	+	+	20	—	20	2,210	428
17	Cut.	1 mo	I	++	+	+	80	20	160	1,070	n.d.
18	Cut.	1 mo	I	+	–	+, RB	—	—	20	1,980	80
19	Cut.	1 mo	I	+	–	–	—	—	320	1,610	303
20	Cut.	1 mo	I	+	+	–	—	—	20	1,200	n.d.

* Lesion form: Cut. = cutaneous, MC = mucocutaneous, n.a. = information not available. Diag. crit. = diagnostic criteria, HE = parasites in histological section, I = parasite isolated from lesion in culture, HCS = history, clinical findings and serology compatible.

† Plasma cell frequency: + = less than 10%, ++ = 10–30%, +++ = 30–50%, ++++ = more than 50% of cells in dermal infiltrates. H&E = haematoxylin & eosin, RB = Russell body.

‡ IFA = antileishmanial indirect fluorescent antibody (reciprocal of titre), DAT = direct agglutination test.

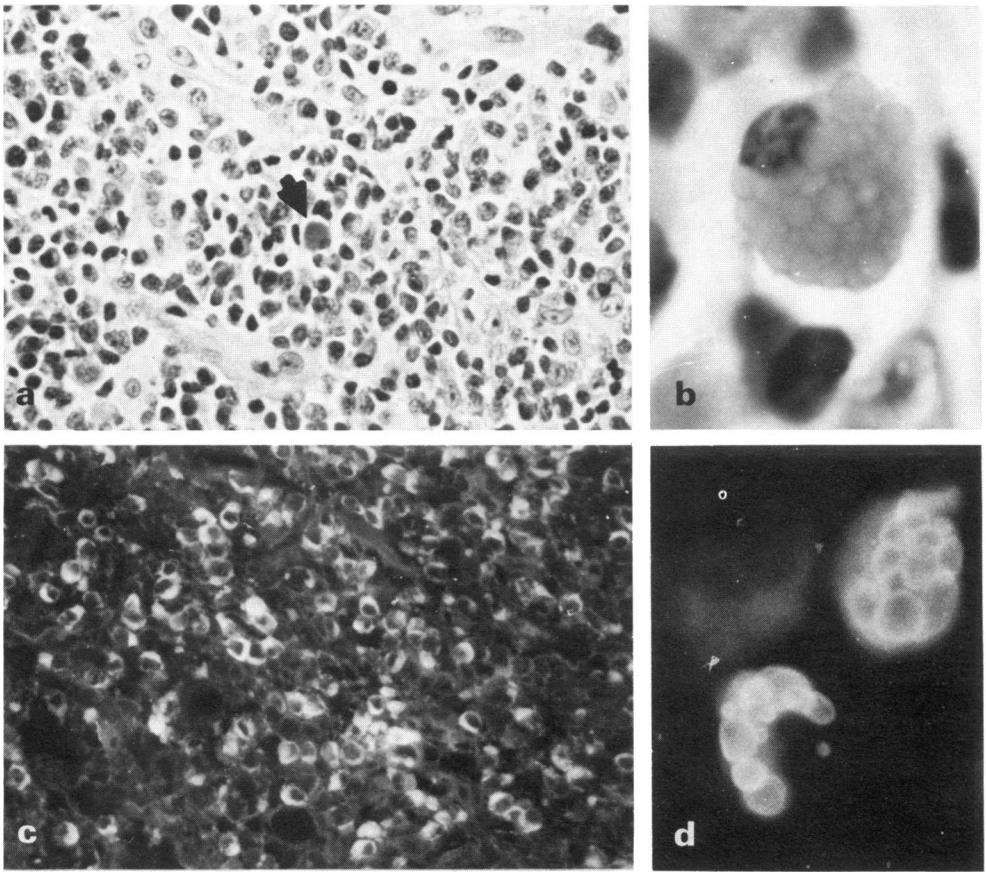

Fig. 1. Plasma cells in human cutaneous leishmaniasis lesions. (a) Inflammatory infiltrate contains numerous plasma cells, including a cell with cytoplasmic Russell bodies (*arrow*). (H&E, Case 4, × 225.) (b) Cytoplasmic Russell bodies of the cell seen in (a) appear as globules. (H&E, × 1,440.) (c) Direct immunofluorescence with gamma-chain-specific anti-human IgG serum revealed that most intralesional plasma cells contain IgG (see Table 1). (Case 6, × 225.) (D) Plasma cells containing Russell bodies showed fluorescence almost exclusively with mu-chain-specific anti-IgM serum (see Table 1). (Case 4, × 1,400.)

When examined by immunofluorescence, there was excellent agreement between the total number of plasma cells in H&E sections and the number fluorescing with anti-IgG antibody, indicating that the majority of plasma cells in the lesions were producing IgG-class immunoglobulins (Table 1, Fig. 1). Cells fluorescing for IgM were found in 17 cases, but were usually present in smaller numbers than those positive for IgG. Rare IgA-producing cells were found in only nine of 18 cases examined with anti-IgA serum. While IgG-fluorescing cells were more frequent, such cells containing globular fluorescent cytoplasmic Russell bodies were observed in only one case, whereas IgM-fluorescing Russell bodies were seen in seven biopsies (Fig. 1). Occasionally, an IgM-fluorescing cell contained a single solidly fluorescent intranuclear body; these were not classed as Russell bodies.

Serological responses

Antileishmanial antibodies were detected by indirect immunofluorescence in 14 sera using anti-IgG antiserum and in seven cases using anti-IgM (Table 1), while all sera were negative with anti-IgA. There was no correlation between the frequency of intralesional plasma cells and the titres of circulating antileishmanial antibodies of the same globulin class. Promastigote agglutinins were detected in 19 cases but exceeded control levels (i.e. $\geqslant 160$) in only six sera (Table 1); all agglutinin

reactions were completely reversed by 2-mercaptoethanol. Precipitin lines on countercurrent immunoelectrophoresis were detected only in cases 5 and 13.

No striking or consistent alterations in complement components were detected, CH50, C3, C4 and B levels being normal or occasionally slightly elevated in 13 cases examined. A low level of circulating immune complexes (17·9% binding) was detected in only one case (No. 1) of 13 tested. Total serum immunoglobulin levels were normal to moderately elevated, but there was no correlation between total globulin levels and frequency of intralesional plasma cells of the same globulin class. IgG exceeded 1,800 mg/dl in seven cases, IgM exceeded 200 mg/dl in six cases (Table 1), while IgA was within normal limits (100–300 mg/dl) in 12 sera, elevated in four and reduced in one serum.

DISCUSSION

The present study has confirmed that plasma cells represent a consistent feature of cutaneous leishmaniasis lesions and has demonstrated that the majority of such cells are producing IgG immunoglobulins. The lack of correlation between frequency of intralesional plasma cells and titres of circulating antileishmanial antibody indicates that serum antibody levels do not provide an adequate estimate of the potential contribution of humoral factors in protection or pathogenesis in this disease. Since it has been estimated that a single plasma cell can produce thousands of immunoglobulin molecules per second (Mitchell, 1979), local antibody concentrations might reach extremely high levels in the immediate vicinity of these cells. Serum antibody levels may be influenced by both local antibody consumption and by extralesional antibody production; thus it is not surprising that a close correlation between intralesional plasma cell numbers and serum antibody levels was not observed.

That plasma cells and their products may play significant roles in both elimination of parasites and in pathogenesis within leishmanial lesions is suggested by a recent histopathological analysis of human leishmaniasis lesions. Ridley (1979) noted an inverse relationship between the frequency of plasma cells and lymphocytes and the number of leishmania within lesions, whereas there was a significant positive correlation between presence of necrosis and intensity of plasma cell and lymphocyte infiltration. Since parasites were never abundant in the present series of biopsies it was not possible to confirm this finding. Detailed histopathological analysis of these lesions will be the subject of a separate communication.

While it might be supposed that intralesional plasma cells would produce antileishmanial antibody, there is no evidence that this is the case. The possibility cannot be excluded that there occurs on a local level within the cutaneous leishmaniasis lesion a polyclonal stimulation of IgG-producing cells similar to that which apparently occurs systemically in visceral leishmaniasis. No attempt to determine specificity of immunoglobulin was made with the formalin-fixed material examined by us.

One interesting but unexplained feature of the present series was the paradoxical finding that Russell bodies were confined almost exclusively to the less frequent IgM-producing cells. These cytoplasmic globules, which are thought to represent intracellular immunoglobulin accumulated due to derangement of normal secretory activity of the cell, are found in both neoplastic and non-neoplastic diseases (Blom, Mansa & Wiik, 1976) but their significance is not clear. In our cases, there was no obvious relation between the presence of Russell bodies and any other feature investigated.

Though it is becoming increasingly clear that the role of humoral factors in cutaneous leishmaniasis has probably been underestimated, the exact nature of that role remains to be determined. Our findings indicate that important humoral mechanisms would most probably involve immunoglobulins of the IgG class. However, further studies, to determine for example the specificity and subclass of intralesional immunoglobulins, are needed to elucidate completely the role of antibodies in protection and pathogenesis of this disease.

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REFERENCES

BLOM, J., MANSA, B. & WIIK, A. (1976) A study of Russell bodies in human monoclonal plasma cells by means of immunofluorescence and electron microscopy. *Acta Pathol. Microbiol. Scand.* **84,** 335.

GRIMALDI, G., JR, MORIEARTY, P. & HOFF, R. (1980) *Leishmania mexicana*: immunology and histopathology in C3H mice. *Exp. Parasitol.* **50,** 45.

HUANG, S.N., MINASSIAN, H. & MORE, J.D. (1976) Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. *Lab. Invest.* **35,** 383.

MANCINI, G., CARBONARA, A. & HEREMANS, J. (1965) Immunochemical quantification of antigen by single radial immunodiffusion. *Immunochemistry*, **2,** 235.

MAYER, M.M. (1961) Complement and complement fixation. In *Experimental Immunochemistry* 2nd edn (ed. by E. A. Kabat and M. M. Mayer). Charles C. Thomas, Springfield, Illinois.

MITCHELL, G.F. (1979) Responses to infection with metazoan and protozoan parasites in mice. *Adv. Immunol.* **28,** 451.

PESSOA, S.B. & BARRETTO, M.P. (1944) *Leishmaniose Tegumentar Americana.* Ministério da Educação e Saúde, Serviço de Documentaçao, São Paulo, Brazil.

POULTER, L.W. (1980) Mechanisms of immunity to leishmaniasis. I. Evidence for a changing basis of protection in self-limiting disease. *Clin. exp. Immunol.* **39,** 14.

PRESTON, P. & DUMONDE, D.C. (1976a) Immunology of clinical and experimental leishmaniasis. In *Immunology of Parasitic Infections* (ed. by S. Cohen and E. Sadun), Blackwell, Oxford.

PRESTON, P.M. & DUMONDE, D.C. (1976b) Experimental cutaneous leishmaniasis. V. Protective immunity in subclinical and self-healing infection in the mouse. *Clin. exp. Immunol.* **23,** 126.

QUALMAN, S.S. & KEREN, D.F. (1979) Immunofluorescence of deparaffinized, trypsin-treated renal tissues. Preservation of antigens as an adjunct to diagnosis of disease. *Lab. Invest.* **41,** 483.

RIDLEY, D.S. (1979) The pathogenesis of cutaneous leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* **73,** 150.

SCHMUNIS, G.A. (1978) *A resposta imune humoral na infecção humana recente pelo* Trypanosoma cruzi. PhD thesis, Universidade Federal do Rio de Janeiro.

VATTUONE, N.H. & YANOVSKY, J.F. (1971) *Trypanosoma cruzi*: agglutination activity of enzyme treated epimastigotes. *Exp. Parasitol.* **30,** 349.

ZUBLER, R.H., LANGE, G., LAMBERT, P.H. & MIESCHER, P.A. (1976) Detection of immune complexes in unheated sera by a modified ^{125}I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. *J. Immunol.* **166,** 232.