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Do infections induce monoclonal immunoglobulin components?

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SUMMARY

The incidental finding of monoclonal immunoglobulin components (MC) in some infections prompted us to study this phenomenon more systematically. Using isoelectric focusing with immunoblotting (detection limit for MC 0·1 mg/ml), the following infections were studied for the presence of MC: visceral leishmaniasis, cytomegalovirus (CMV) infection, echinococcosis and infectious mononucleosis. MC were found in 16 of 20 leishmania patients and in eight of 18 CMV patients, but in only one of 20 echinococcosis patients and in none of 30 infectious mononucleosis patients. The MC were mostly transient, where tested. A minority of the MC found in the leishmaniasis patients was shown to bind to leishmania antigens. The specificity of the majority of the MC remains unknown. Further study is required to explain the high incidence of MC in CMV infection and visceral leishmaniasis.

Keywords monoclonal immunoglobulin components visceral leishmaniasis cytomegalovirus mononucleosis

INTRODUCTION

Monoclonal immunoglobulin components (MC) are structurally uniform antibody populations in serum produced by expanded B cell clones. Originally, MC have been found in malignant B cell disorders, e.g. multiple myeloma and Waldenström's macroglobulinaemia, where they may be present at a high concentration and are usually termed paraproteins. With the increased sensitivity of the methods employed, MC were also found in a number of other conditions, mainly in age-related benign B cell proliferative disorders and in certain immunodeficiencies.

The development of MC in experimental infections in animals has been reviewed (Nisonoff, Hopper & Spring, 1975; Krause & Kindt, 1977). A possible role for infections in inducing MC in humans has been suggested (Isobe & Osserman, 1971). A number of observations of MC in patients with certain infections (Vodopick *et al.*, 1974; Bussel *et al.*, 1978; Groshong *et al.*, 1976; Oxelius, 1972) supported this view. Recently, a high association of HIV infection with MC has been found (Papadopoulos *et al.*, 1985; Crapper, Deam & Mackay, 1987). For other infections such a high association has not been demonstrated.

Using isoelectric focusing (IEF) with immunoblotting as a sensitive technique for detecting MC (Haas, Lange & Schlaak, 1987), we studied four infections for the presence of MC: visceral leishmaniasis, cytomegalovirus (CMV) infection, echinococcosis, and infectious mononucleosis. Since MC are found also in individuals with increasing age, without any overt disease—the so called benign monoclonal gammopathy (Radl, 1986)—only young patients were included in this study.

Among the four infections studied, MC were found at a high frequency in CMV mononucleosis and visceral leishmaniasis.

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PATIENTS AND METHODS

Patients

Twenty patients with visceral leishmaniasis (Caucasian residents of FRG who had been infected during an overseas journey), 18 patients with clinically apparent CMV infection (i.e. CMV mononucleosis), 20 patients with *Echinococcus granulosus* infection, and 30 patients with infectious mononucleosis were included in this study. In the CMV patients AIDS was excluded (negative HIV serology); the other patients were not tested for HIV. All patients were younger than 35 years (Table 1).

Thirty-nine blood donors aged 17 to 35 years (mean 25) served as controls.

Reagents

Alkaline phosphatase (AP) conjugated, affinity-purified $F(ab')_2$ goat anti-human IgG, Fc-specific; AP-conjugated, affinity-purified goat anti-mouse IgG, AP-conjugated goat anti-rabbit IgG, biotinylated human IgG (Jackson Immunoresearch, distri-

Table 1. Frequency of monoclonal immunoglobulin components (MC)

Patients	total	MC^+		Age (years)*
		n	%	
Leishmaniasis	20	16	80	10
CMV infection	18	8	44	26
Echinococcosis	20	1	5	26
Infectious mononucleosis	30	0	0	11
Controls	39	1	3	25

* Mean age (range 1–35)

buted by Dianova, Hamburg, FRG); rabbit anti-human IgA and rabbit anti-human IgM, heavy chain specific (Dako). Mouse monoclonal antibodies to human IgG subclasses: IgG1, clone NL16 (Unipath); IgG2, clone HP6014 (ICN); IgG3, clone NI86 (Nordic); IgG4, clone RJ4 (Unipath). Goat anti-human kappa and lambda chain antisera (Behring) were used biotinylated. AP-conjugated streptavidin (Jackson Immunoresearch).

Tris–Tween buffer: 0·1 M Tris-Hcl, pH 7·5, containing 0·1 M NaCl, 2 mM $MgCl_2$, and 0·05% (v/v) Tween 20.

AP-9·5 buffer: 0·1 M Tris-HCl, 0·1 M NaCl, 5 mM $MgCl_2$, pH 9·5.

Phosphate buffered saline (PBS): pH 7·2, 0·15 M; 0·14 M NaCl, 2·6 mM KCl, 80 mM Na_2HPO_4, 14 mM KH_2PO_4.

Preparation of leishmania antigen

This was performed as described in detail elsewhere (Mannweiler, Lederer & Zum Felde, 1978). Briefly, by repeated freeze–thawing and ultrasonication an aqueous extract was obtained from a mixture of *Leishmania donovani*, *L. brasiliensis* and *L. tropica* (reference strains kept at the Bernhard Nocht Institute, Hamburg, FRG).

Affinity chromatography

The immunosorbent was prepared by coupling antigen to CNBr-activated sepharose 4B (Pharmacia) according to the manufacturer's instructions. Leishmania antigen (1 mg) was bound to 200 μl of gel, a column was prepared and equilibrated with PBS. After applying 20 μl of serum and extensive washing with PBS, specific antibody was eluted with glycine–HCl, pH 2·5, 10% dioxane and immediately dialysed against PBS.

Immunoelectric focusing

IEF was run in 0·8% agarose gel (Agarose IEF, Pharmacia) containing an ampholyte mixture of 2% (w/v) ampholines 3·5–10 (LKB) and 2% (w/v) pharmalytes 3–10 (Pharmacia) (H.I. Schipper, Göttingen, personal communication). Prior to application, serum was diluted 1:100 in PBS (in case of high IgG content up to 1:250). The protein concentration (OD at 280 nm) of the effluent ('wash') of the affinity column was 100–700 μg/ml, and that of the eluate 50–100 μg/ml. The samples were applied at a volume of 10 μl/cm. The application site was 2 cm from the anode, i.e. at acid pH. The current was limited to 20 mA, the voltage to 2000 V, and the power to 35 W. The temperature of the coolant was set at 0°C. The duration of a run was about 70 min (1200 Vh).

We found that the use of ampholines and pharmalytes instead of servalytes, that we had used before, improved the detection of MC. A possible explanation for this may be a more continuous pH gradient of these ampholytes. Application of the samples at the acid pH site further enhanced the sensitivity (data not shown).

Immunoblotting

Using the print technique, the separated serum components were transferred from the agarose gel to a nitrocellulose membrane (BA 85; Schleicher & Schüll). After blocking the remaining free binding sites with Tris–Tween buffer, the membrane was cut into 0·3-cm wide strips. These were placed into multi-channel plates (eight-channel reservoir, Flow Lab.) and incubated with anti-heavy and anti-light chain antibodies diluted in Tris–Tween buffer (dilutions of monoclonal antibodies ranging from 1:10000 to 1:50000, and of polyclonal antibodies from 1:15000 to 1:80000). Strips were washed with Tris–Tween buffer and, if the first antibody was not enzyme-labelled or was biotinylated, incubated with the AP-conjugated second antibody or AP-conjugated streptavidin. After repeatedly washing, the membrane strips were developed by an enzymatic reaction according to Leary, Brigati & Ward (1983). The reaction was stopped by briefly washing in distilled water and drying. For total protein staining India ink was used (Hancock & Tsang, 1983). To detect the rheumatoid factor activity of MC, strips were incubated with biotinylated human IgG, washed, and then incubated with AP-conjugated streptavidin, washed and developed.

Criteria to identify MC

The following three criteria were used: (i) MC are restricted to one heavy and one light chain type; (ii) due to their microheterogeneity, MC form not only one but several narrow-spaced bands (Righetti & Drysdale, 1974); and (iii) under the IEF conditions used, MC show sharp distinct bands compared with polyclonal immunoglobulins which show diffuse bands.

Sensitivity of IEF with immunoblotting

To assess the sensitivity of IEF with immunoblotting for detecting MC of the IgG class, serial dilutions of a purified IgGl/λ paraprotein in normal human serum were tested. The detection limit was found to be about 0·1 mg paraprotein/ml of serum (Fig. 1). The sensitivity for other paraproteins was: IgG1/κ 0·125 mg/ml; IgG2/λ <0·1 mg/ml; IgG3/λ 0·25 mg/ml; IgG4/λ 0·03 mg/ml; IgM/κ 1·0 mg/ml; IgA/λ 0·5 mg/ml. These values do not necessarily represent the real sensitivity of the procedure, since they depend on the quality of the purified paraproteins. The purified IgG3 and IgA paraproteins were heavily aggregated and showed a diffuse banding pattern which negatively affects the detection.

RESULTS

MC in healthy individuals

Since MC are found also in apparently healthy individuals in an age-dependent manner (Radl, 1986), we included in our study a control group of 39 young blood donors aged 17 to 35 years (mean 25). Thirty-eight of the 39 young donors did not show MC (Fig. 2a; Table 1).

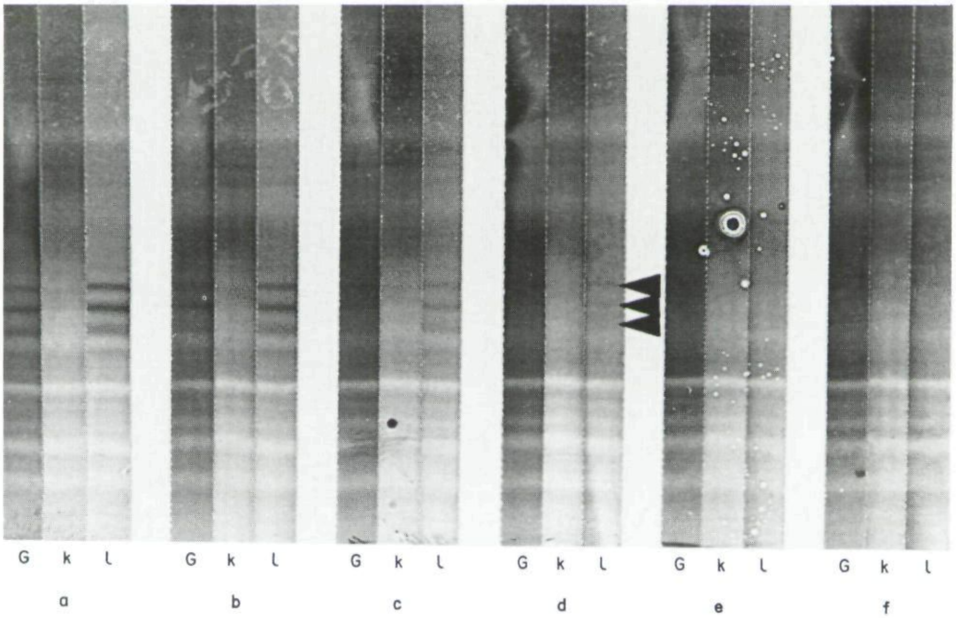

Fig. 1: Sensitivity of IEF with immunoblotting for detecting MC. An IgG1/λ paraprotein was serially diluted with normal human serum, separated by IEF, blotted onto a nitrocellulose membrane and developed with antibodies to γ, κ and λ chains (G, k, l). The dilutions were: (a) 0·5; (b) 0·25; (c) 0·125; (d) 0·062; and (e) 0·031 mg of paraprotein per ml of normal human serum; (f) pure serum. The limit for detecting monoclonal IgG is ≤0·1 mg/ml (arrows).

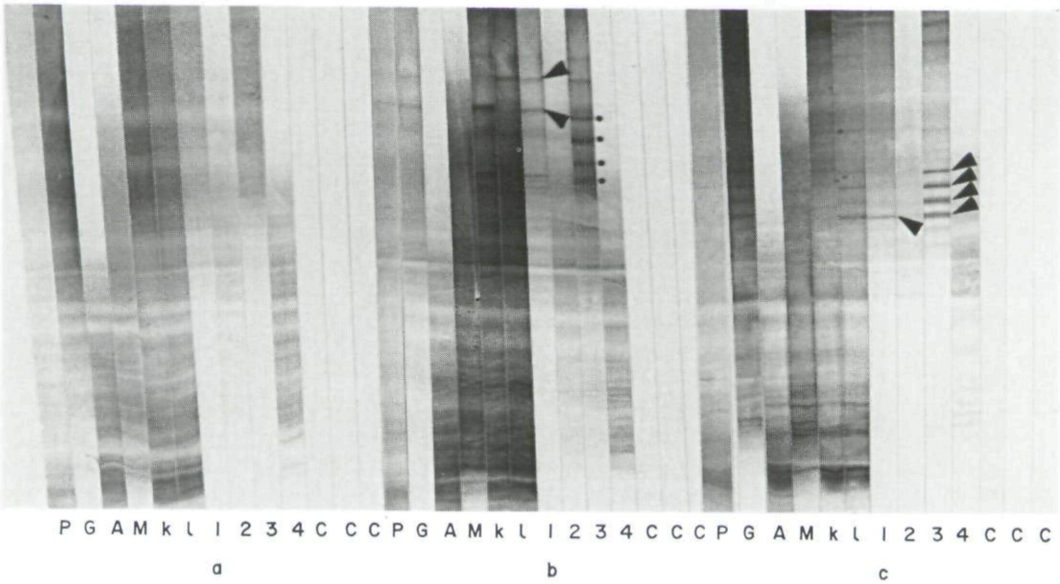

Fig. 2. Immunoblot analysis of a normal human serum without MC (a) and two CMV patients' sera with MC (b, c). Serum was separated by IEF, blotted onto nitrocellulose membrane and developed with India ink (P) and antibodies to heavy and light chains, including IgG1–4 (G, A, M, k, l, 1, 2, 3, 4); buffer controls (C). MC were found in (b): IgG1/κ (upper arrow), IgG1/λ (lower arrow); and in (c): IgG3/λ (upper arrows) and IgG1/λ (lower arrow), the latter sharing the pI with the lowermost IgG3/λ band. MC form distinct bands (b, c), whereas polyclonal immunoglobulins appear as diffuse bands (a). (The clonotypic IgG3 bands in (b) (points) do not find corresponding light chain bands and are thus not considered MC).

Only one donor, aged 34 years, showed a small MC component. A follow-up serum taken 17 months later showed an identical result. We could not obtain data on the health status of this donor. Thus, it was not possible to explain the finding.

MC in patients

To exclude MC due to age-related proliferative disorders, only young patients (≤35 years) were included in this study. As shown in Table 1, the frequency of MC differed among the four groups investigated; we found a high incidence in leishmaniasis (80%) and CMV infection (44%; examples in Fig. 2) and a low incidence in echinococcosis (5%) and infectious mononucleosis (0%).

In several patients more than one MC was observed. Regarding the isotype, MC were only of the IgG class. However, we cannot exclude that also monoclonal IgA and IgM compo-

Table 2. IgG subclass and light chain distribution of monoclonal immunoglobulin components (MC)

Patients	IgG1	IgG2	IgG3	IgG4	κ	λ
Leishmaniasis	22	1	2	0	11	14
CMV mononucleosis	10	0	2	0	5	7
Echinococcosis	0	1	0	0	0	1
Infectious mononucleosis	0	0	0	0	0	0
Controls	1	0	0	0	1	0

In several patients more than one MC was found

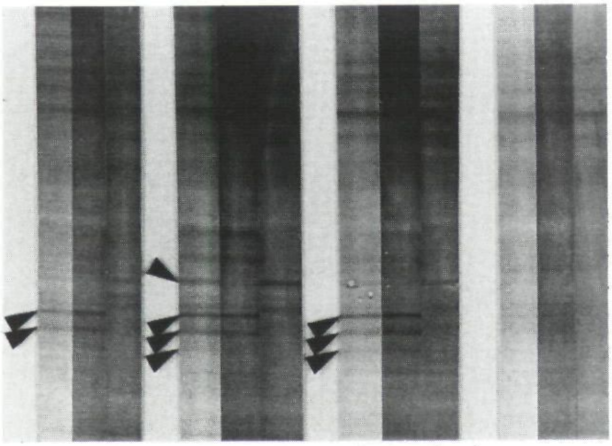

Fig. 3. Longitudinal observation of an individual patient. From a leishmania patient several sera obtained prior to and during therapy were subjected to immunoblot analysis; (G) IgG1, (k) κ, and (l) λ. The samples were obtained (a) on admission, (b) 1, (c) 7, and (d) 19 months after admission, respectively. In (a), (b), and (c) a monoclonal IgG1/κ (lower arrows) and in (b) a monoclonal IgG1/λ (upper arrow) are seen, whereas (d) does not show MC. Thus, MC are transient in this patient.

nents are produced in these infections, considering that our technique is optimal for the detection of monoclonal IgG, but less suitable for the detection of a low level of monoclonal IgA and IgM (data not shown). Regarding the subclass distribution, most MC were IgG1. In addition, we found some monoclonal IgG3 and IgG2, but we did not observe monoclonal IgG4 (Table 2).

It has been established that due to its microheterogeneity, a paraprotein focuses into several regularly spaced bands, forming the so-called clonotype. However, the lower the concentration of a paraprotein, the fewer bands of its clonotype are seen (Sinclair, Parrott & Stott, 1986; Haas *et al.*, 1987; Hamilton *et al.*, 1987). In our sera, several MC were represented by only one or two bands, thus reflecting a low concentration of the respective MC.

Follow-up of MC in individual patients

In order to determine whether MC remain or disappear during reconvalescence, we studied follow-up sera from seven patients (four with leishmaniasis and three with CMV) over a period of up to 4 years. In six of the seven patients MC disappeared after several months (example in Fig. 3), and the maximum duration of MC was 18 months. In one patient the MC did not disappear during the observation period; however, the follow-up was possible for only 12 months. Thus, the data suggest that MC in these diseases are usually transient.

Specificity of MC

Using affinity chromatography with leishmania antigen coupled to sepharose, we analysed all 20 leishmaniasis sera. In parallel, the native serum, the unbound fraction, and the fraction binding to the immunosorbent were analysed for MC (Fig. 4). We found a higher number of MC in the unbound fraction than in the eluate (Table 3).

To test MC for rheumatoid factor RF activity, the binding of labelled human IgG to MC was studied. All MC were RF negative.

DISCUSSION

Apart from HIV, no infection has been shown to be frequently associated with MC. Here we demonstrate that MC are closely associated with visceral leishmaniasis and CMV infection. As a technical tool we used IEF with immunoblotting, which has a sensitivity for detecting monoclonal IgG components in serum of about 0·1 mg/ml (Fig. 1). The low incidence of MC reported previously can be explained by the low sensitivity of the detection methods employed; they revealed only the tip of the iceberg.

It should be mentioned, however, that in using antigen-specific methods, usually based on radiolabelled antigens, many investigators found a restricted clonality of human antibody responses to bacterial antigens (Insel, Kittelberger & Anderson, 1985; Morrow, Macy & Stevens, 1981; Shackelford *et al.*, 1988). This restriction does not necessarily imply the presence of MC, since the term MC ('paraprotein') is relative: it is restricted to monoclonal immunoglobulins present at a high concentration compared with the background of the residual immunoglobulins. Antigen-specific methods are certainly more sensitive than antigen-non-specific methods, but they do not consider the background of unrelated immunoglobulins. This background is assessed only by antigen-non-specific methods such as immune electrophoresis, immunofixation or the immunoblot approach used in this study.

The frequency of MC differed with the infections investigated. We found a high frequency in leishmania patients (80%) and in CMV patients (44%). To our knowledge, an association between leishmaniasis and MC has not been reported before, whereas the high incidence of MC in CMV patients is in agreement with observations of paraproteins during CMV infection (Bussel *et al.*, 1978; Vodopick *et al.*, 1974). On the other hand, a low frequency of MC was observed in echinococcosis. The complete absence of MC in infectious mononucleosis was surprising for us, since this disease is caused by the Epstein–Barr virus (EBV) which is a B cell activator and is associated with B cell lymphomas. However, we have found MC in persons with chronic EBV infection (not included in this study): in one of two young patients with X-linked lymphoproliferative disease and in two of three apparently healthy young persons with unusually high antibody titres to EBV viral capsid antigen

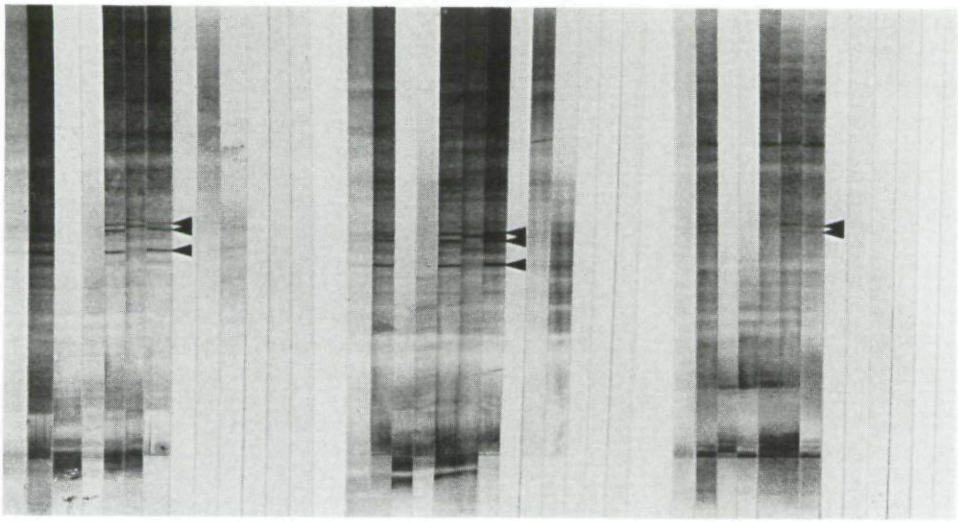

Fig. 4. Specificity of MC. A leishmania patient's serum was separated by affinity chromatography with leishmania antigen coupled to sepharose. The native serum, the effluent ('wash') and the eluate from the column were analyzed in parallel (for details see Fig. 2). A small monoclonal IgG1/λ is selectively seen in the eluate (right arrows) whereas the prominent IgG1/κ (left and middle arrows) is not. This suggests that the IgG1/λ is directed to the leishmania antigen, whereas the IgG1/κ is not.

Table 3. Affinity chromatography of leishmania sera: monoclonal immunoglobulin components (MC) were found in the unbound and the bound fraction

	Unbound fraction (wash)	Bound fraction* (eluate)
IgG1/κ	11	2
IgG1/λ	11	7
IgG2/κ	1	—
IgG3/κ	1	—
IgG3/λ	—	1

* Presumably due to enrichment some MC appeared in the bound fraction that were not visible in the native serum.

(VCA) and early antigen (EA). This suggests the involvement of additional factors.

The specificity of MC to the infectious agent was studied using affinity chromatography and the sera of the leishmania patients. For this purpose a broad leishmania antigen preparation for routine diagnostics was coupled to sepharose. Only a minority of MC bound to this immunosorbent. This corresponds to the observation that the bulk of increased IgG in leishmania patients is not directed to the parasite (Behin & Louis, 1984). Moreover, MC did not show RF activity which is frequently found in leishmania patients. This suggests that MC were not involved in the clearance of immune-complexed parasite antigens.

The proposition that the MC observed in this study were induced by infection is supported by the following: (i) MC due to aging were excluded, since we studied only young patients; (ii) MC due to additional disorders were excluded, since we studied only patients with a single disease; (iii) it is unlikely that MC were induced by the medical treatment, since they were already present prior to therapy in several cases; and (iv) the longitudinal study of some patients showed that MC disappeared with improvement of the clinical symptoms.

What are the mechanisms leading to the production of MC? As is known from studies on immunodeficiencies, transplantation patients and various animal models, the production of many MC is closely associated with T cell defects (Radl, 1979; van den Akker *et al.*, 1988; Ghory, Schiff & Buckley, 1986). MC can even be used as indicators of regulatory T cell defects (Radl *et al.*, 1985). Indeed, visceral leishmaniasis is characterized by a profound T cell depression (Sacks *et al.*, 1987), and CMV infection suppresses cellular immunity in humans and in mice (Rook, 1988; Howard, Miller & Najarian, 1974). In contrast, a defective function of T lymphocytes is found only transiently in infectious mononucleosis (Junker *et al.*, 1986) and, to our knowledge, not at all in echinococcosis. In the latter, a preserved cellular reactivity to the parasite is of diagnostic significance (Casoni test).

The duration of the infection might also contribute to the production of MC. As known from animal studies, chronic antigenic stimulation with bacterial cell wall components leads to the formation of MC (Krause, 1970). Nevertheless, our data indicate that chronicity is not the decisive factor: echinococcosis is a chronic disease, but only one of 20 patients showed MC.

The same applies to hypergammaglobulinaemia: all four infections under study were characterized by hypergammaglobulinaemia, but only two were associated with MC.

Certain infections are frequently associated with the production of MC. It is possible that the depressed cellular immunity, and consequently a temporary defect in the regulatory circuits, is the major factor leading to the high incidence of MC in leishmaniasis and CMV infection. Further studies are needed to elucidate the operation of these mechanisms.

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