Clin. exp. Immunol. (1980) **39**, 289–296.

Lymphocytotoxins in leprosy and in asymptomatic hepatitis B virus infection

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(*Accepted for publication* 13 *August* 1979)

SUMMARY

Serum lymphocytotoxic antibodies (LCAs) were detected in 67% of Papua New Guinean lepromatous leprosy patients who were persistent carriers of hepatitis B surface antigen (HBsAg). Lymphocytotoxins were not associated with asymptomatic HBsAg in either healthy controls or tuberculoid leprosy patients. It was apparent that, although HBsAg itself is a poor indicator of *in vitro* lymphocytotoxicity, when the antigen occurred in a host with impaired immune response, lymphocytotoxicity was enhanced. In contrast to this finding, lepromatous leprosy patients without HBsAg had significantly depressed LCA production in comparison with tuberculoid patients and controls. The interaction between leprosy and hepatitis B virus was highly significant ($P = 0{\cdot}001$) in an analysis of variance of cytotoxicity scores. It is proposed that the previously reported equivocal results regarding autoantibodies in leprosy patients may be explained by this unusual interaction between lepromatous leprosy and hepatitis B virus infection.

INTRODUCTION

An increased prevalence of lymphocytotoxic sera has been observed in patients with acute viral infection (Mottironi & Terasaki, 1970) when compared with sera from healthy controls. An even higher prevalence of cytotoxicity is observed in sera from patients with autoimmune diseases including systemic lupus erythematosus (SLE) (Terasaki, Mottironi & Barnett, 1970; Winfield, Winchester & Kunkel, 1975) and rheumatoid arthritis (Naito *et al.*, 1971). Cold-reacting lymphocytotoxic antibodies (LCAs) have been confirmed also in other idiopathic diseases such as multiple sclerosis (Shocket & Weiner, 1978) and inflammatory bowel disease (Korsmeyer *et al.*, 1975; Strickland *et al.*, 1975).

The biological stimulus for LCA production remains obscure. Tissue damage *per se* in chronic and acute hepatitis has been favoured as a causative mechanism by DeHoratius, Henderson & Strickland (1976) while Ooi *et al.* (1974) found a positive association between the nature of renal involvement and LCAs in SLE. However, the increased incidence of LCAs in non-consanguineous relatives of patients with SLE (DeHoratius & Messner, 1975), inflammatory bowel disease (Korsmeyer *et al.*, 1975) and multiple sclerosis (Shocket & Weiner, 1978) suggests that LCAs may be induced by environmental agents.

This study examines whether lymphocytotoxins are related directly to an infective agent or are modified by the immune response of the host. The incidence of LCAs was determined in leprosy patients and their controls. Although the infective agent in all types of leprosy is *Mycobacterium leprae*, the clinical expression of the disease is modified by the host's immune status. Lepromatous patients may suffer

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0099-9104/80/0200-0289$02.00 © 1980 Blackwell Scientific Publications

damage to vital organs while tuberculoid leprosy patients have dermal or neural involvement. The erythema nodosum leprosum (ENL) of some lepromatous patients has been likened to autoimmune collagen-vascular diseases, particularly SLE (Wager, 1969) which has the highest rate of LCAs yet reported.

Autoantibodies in leprosy patients have been recorded previously by Bonomo & Dammacco (cited in Petchclai *et al.*, 1973) who found a high prevalence of rheumatoid factor, anti-thyroglobulin antibody and anti-nuclear antibody in lepromatous patients. Since rheumatoid factor and anti-nuclear factor are correlated positively with LCAs in autoimmune diseases such as SLE and rheumatoid arthritis (Terasaki *et al.*, 1970), we have compared the prevalence of LCAs in lepromatous patients with that in healthy controls.

Lepromatous and tuberculoid leprosy patients and controls were selected to include comparable numbers with asymptomatic carriage of hepatitis B surface antigen (HBsAg), since lepromatous patients generally have a high rate of persistent HBsAg (Blumberg *et al.*, 1967) and hepatitis B virus (HBV) itself may be related to LCA production. The question of whether HBsAg carriage is associated with lymphocytotoxins is not yet resolved, for although DeHoratius *et al.* (1976) did not detect LCAs in ten persons with asymptomatic HBsAg Kingham *et al.* (1978) reported DNA-binding antibodies in 95% of those with HBsAg. As DNA-binding antibodies and LCAs are correlated positively in SLE (Ooi *et al.*, 1974), this study also re-examines the possible relationship between lymphocytotoxins and HBsAg in a larger sample.

PATIENTS AND METHODS

Patients. Patients and controls from the north Papua New Guinea mainland coast had previously been examined for leprosy and hepatitis B virus (HBV) status as described elsewhere (Serjeantson & Woodfield, 1978; Serjeantson, Wilson & Keats, 1979). Age, sex, parity and institutional history were also recorded. From sera stored at −80°C, 165 specimens were selected for assessment of lymphocytotoxins. The sample included sixty-two lepromatous, nine borderline lepromatous and forty-three tuberculoid patients, with fifty-one controls with no history of leprosy. The sample was selected to include approximately comparable proportions of persons with HBsAg (25%) or its antibody (30%) within each leprosy type. Throughout the analysis, lepromatous and borderline lepromatous groups are combined.

Methods. Lymphocytotoxic antibodies were measured by the microdroplet method of Terasaki & McClelland (1964), modified for testing at 15°C. Target cells used in the microtoxicity assay were peripheral B lymphocytes from twelve individuals of Caucasian, Indian and Chinese origin as well as lymphocytes from ten chronic lymphatic leukaemia patients with known HLA-A, -B and DRw specificities. The majority of lymphocytotoxic sera recognize antigens on both B and T peripheral lymphocytes at 15°C (Strickland *et al.*, 1975), but a small proportion of sera are specific for T or B cell subpopulations. Therefore, we have confined our target cells to B lymphocytes only.

Normal lymphocytes were separated from 20 cm^3 peripheral venous blood by Ficoll–Hypaque gradient centrifugation. After washing, polymorphonuclear cells were removed by carbonyl ion depletion method using a lymphocyte separating agent (Technicon Products New York, NY). B lymphocytes were isolated by the removal of the E-rosette forming population based on the method of Pellegrino, Ferrone & Theofilopoulos (1976) with modifications as described. Lymphocyte suspension (3 cm^3) was mixed with an equal volume of foetal calf serum (FCS) containing a 1% suspension of sheep red blood cells coated with amino-ethylthiouronium bromide (AET). The mixture was incubated for 15 min at 37°C before light centrifugation and overnight incubation at 4°C. Gentle resuspension of cells enabled the extent of E-rosette formation for each preparation to be assessed. E-rosette formation was within a 50 to 85% range depending on the particular donor. Ficoll–Hypaque was underlayered and upon gradient centrifugation, the B lymphocyte band formed at its interface, separating it from the pellet-forming T lymphocyte subpopulation.

B lymphocytes (2×10^3) were first incubated in each well of a microtitre plate with 0·001 cm^3 of each of one hundred and sixty-five test sera for 1 hr at 15°C. Rabbit complement (0·005 cm^3) was then added to each well and the mixture was incubated at 15°C for a further 2 hr. Target cell death was assessed by the uptake of eosin-Y dye with the use of inverted phase-contrast microscopy. A positive and negative control were included in every test assay.

HBsAg was detected by the 'Auscell' (Abbott Laboratories) reversed passive haemagglutination method and also by immunoelectrophoresis (IEOP). Anti-HBs was tested by passive haemagglutination assay (PHA) following Vyas & Shulman (1970). Presence of anti-HBs antibodies was accepted when there was a titre above 1:8 accompanied by positive inhibition with a standard HBsAg serum.

Cytotoxicity was scored on a five-point scale from 0 to 4, to quantitate the proportion of target cells killed. Reactions were assessed as less than 20% of target cells killed, 20–50%, 50–75%, 75–90% or 90–100%. Mean cytotoxicity scores were determined from the simple mean of twenty-two cell reactions, so that a mean serum cytotoxicity score of 1·0 corresponds to an average killing of 20–50% of lymphocytes in each microlymphocytotoxic test.

RESULTS

The Papua New Guinea sera, including those from healthy controls, were considerably more lymphocytotoxic than those from Caucasians tested in the same laboratory. In the Papua New Guinea series, 21·6% of sera from persons unaffected by leprosy had a cytotoxicity index greater than 1·0 or killed, on average, in excess of 20% of test panel cells. The frequency distributions of cytotoxicity scores for fifty-one Papua New Guineans without leprosy and for 116 Australian blood donors are compared in Fig. 1. Only 2·6% of Caucasian sera attained a cytotoxicity score greater than 1·0. Although ethnic comparisons are not valid in this data set, since the Papua New Guinean sera are not from random controls but from individuals purposefully selected with respect to their HBV status, it seems likely that the tropical sera reflect exposure to a multitude of antigenic stimuli. Not the least of these is malaria, which is known to result in production of a wide variety of red cell autoantibodies (Rosenberg *et al.*, 1973) and parasitic infections such as ascariasis and filariasis which have been implicated in serum lymphocytotoxicity (Mayer, Falkenrodt & Tongio, 1973). Fig. 1 demonstrates that Papua New Guinean cytotoxicity scores approach normality in distribution and that data transformation, as would generally be necessary if Caucasian lymphocytotoxicity scores were to be treated as a quantitative variable, is not required.

Lymphocytotoxicity at 15°C was not related to histocompatibility phenotypes of the twenty-two target cells and this was confirmed by the absence of any age, sex or parity relationship with cytotoxicity. Mean cytotoxicity in ninety-one males was $0{\cdot}57 \pm 0{\cdot}04$ compared with $0{\cdot}58 \pm 0{\cdot}05$ in seventy-four females and with $0{\cdot}59 \pm 0{\cdot}11$ in twenty-four females who reported more than one pregnancy. Mean lymphocytotoxicity was $0{\cdot}65 \pm 0{\cdot}10$ in twenty-three children aged less than 20 years and $0{\cdot}56 \pm 0{\cdot}04$ in 142 adults. Similarly, lymphocytotoxicity was not related to cold auto-haemagglutinins which have been reported (Booth, Jenkins & Marsh, 1966) in a high proportion of Melanesian sera. In our series, 64·2% of fifty-three sera showed cold agglutinin activity at 4°C, 30·2% at 15°C and 13·2% at 20°C when agglutination was read macroscopically. The presence of serum haemagglutinins did not coincide with cold lymphocytotoxicity, with the correlations for joint occurrence of serum red and white cell antibodies being $r = 0{\cdot}004$ at 4°C, $r = 0{\cdot}082$ at 15°C and $r = 0{\cdot}156$ at 20°C.

Table 1 provides mean lymphocytotoxicity scores for lepromatous and tuberculoid leprosy patients and for controls, within each HBV class. The highest mean cytotoxicity occurred in lepromatous patients who were positive carriers of HBsAg and the lowest mean cytotoxicity also occurred in lepromatous patients, but in those with detectable anti-HBs. In the HBsAg positive group, the mean score of $0{\cdot}97 \pm$

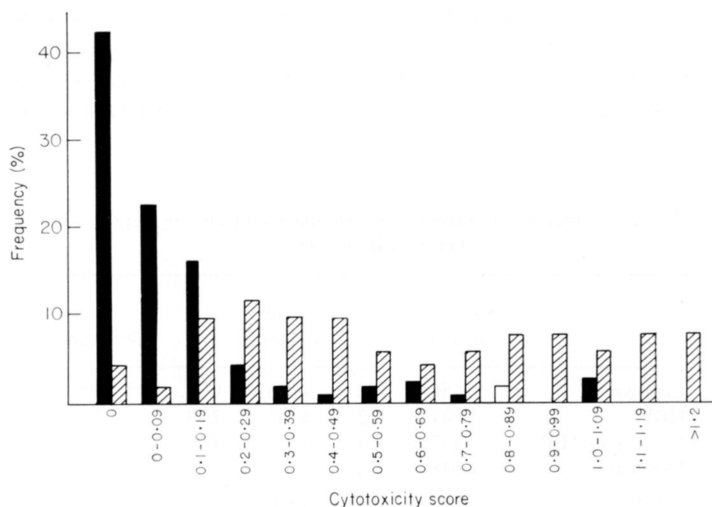

FIG. 1. Frequency distributions of lymphocytotoxicity scores in healthy coastal Papua New Guineans and in Caucasian blood donors. (▨) Papua New Guinean control sera, (■) Caucasian blood bank sera.

TABLE 1. Mean lymphocytotoxicity scores by hepatitis B virus status and leprosy

HBV status		Leprosy status: Lepromatous			Tuberculoid			Controls			t value lepromatous *vs* controls
HBsAg	Anti-HBs	No.	$\bar{X}$	s.e.	No.	$\bar{X}$	s.e.	No.	$\bar{X}$	s.e.	
−	−	33	0·48	0·06	13	0·55	0·11	20	0·58	0·09	−0·57
+	−	15	0·97	0·15	14	0·60	0·13	13	0·53	0·12	2·31*
−	+	20	0·35	0·06	15	0·60	0·12	16	0·76	0·11	−3·46†
+	+	3	—	—	1	—	—	2	—	—	—
Totals		71	0·54	0·05	43	0·58	0·07	51	0·62	0·06	−1·01

* 0·01 < P < 0·05.
† P < 0·01.

0·15 in lepromatous patients was significantly higher ($P<0·05$) than the mean of 0·53±0·12 in controls. Conversely, in the anti-HBs positive group, the mean score of 0·35±0·06 in lepromatous patients was significantly lower ($P<0·01$) than the mean of 0·76±0·11 in controls. Tuberculoid patients had lymphocytotoxic scores intermediate between lepromatous leprosy patients and controls within each of the three HBV classes, but did not differ significantly from controls.

The significance of the interaction between leprosy type and hepatitis B virus status is tested in an analysis of variance of cytotoxicity scores given in Table 2. For purposes of analysis, subjects were categorized as lepromatous, including borderline lepromatous or tuberculoid including borderline tuberculoid, or as unaffected, with respect to leprosy. The same subjects were categorized in three HBV status groups: HBsAg present, or anti-HBs present (including six persons also HBsAg positive), or no serological evidence of exposure to the virus. Table 2 shows that HBV status is related significantly to cytotoxicity. The same analysis also indicates that although leprosy status alone does not influence lymphocytotoxicity, leprosy and hepatitis interact strongly (P = 0·001) to account for a significant proportion of individual variation in cytotoxicity scores.

The significant contribution (P = 0·05) of HBV status to the analysis of variance in cytotoxicity scores is attributable entirely to the influence of the presence or absence of HBsAg on lymphocytotoxicity as shown in Table 3. Presence or absence of anti-HBs was not a determinant of cytotoxicity when leprosy type was ignored. As shown in Table 3, persons with HBsAg had a mean score of 0·71±0·08, significantly higher (t = 2·19, $0·01<P<0·05$) than the mean value of 0·52±0·05 for sixty-six persons showing no serological evidence of exposure to the virus. The lowest mean cytotoxicity score was observed in the

TABLE 2. Analysis of variance of lymphocytotoxicity by leprosy and hepatitis B virus status

	Sums of squares	d.f.	Mean square	f	Significance
Leprosy	0·156	2	0·078	0·46	0·999
HBV	1·004	2	0·502	2·94	0·054
Leprosy by HBV	3·257	4	0·814	4·77	0·001
Residual	26·648	156	0·171	—	—
Total	31·105	164	0·190	—	—

TABLE 3. Mean lymphocytotoxicity scores by hepatitis B virus status

HBV Status		Number	Lymphocytotoxicity		
HBsAg	Anti-HBs	tested	Mean	s.d.	s.e.
−	−	66	0·522	0·365	0·045
+	−	42	0·711	0·534	0·082
−	+	51	0·548	0·423	0·059
+	+	6	0·436	0·366	0·149

small number of individuals with both HBsAg and anti-HBs in their serum. The impact of HBsAg on cytotoxicity, although statistically significant, is not dramatic and this may be due, in part, to the wide variety of antigenic stimuli in tropical coastal areas causing a high background of lymphocytotoxicity. Even so, HBsAg has increased mean cytotoxicity by 41% in this population.

In Table 4, frequency distributions of lymphocytotoxicity scores in lepromatous patients are compared with the non-lepromatous group. The lepromatous leprosy and HBV interaction is readily apparent if an arbitrary definition of 'cytotoxicity' is provided, such that a score greater than 0·8 describes a serum as lymphocytotoxic. One third of all sera tested were cytotoxic under this definition. Then in the HBsAg positive group 67% of lepromatous sera contained lymphocytotoxins in contrast to 26% of the non-lepromatous sera with $\chi_1^2 = 6{\cdot}64$, $P = 0{\cdot}01$, for the comparison. In the fifty-three lepromatous patients without HBsAg, only 11% of sera were cytotoxic compared with 42% of sixty-four non-lepromatous sera with $\chi^2{}_1 = 13{\cdot}64$, $P<0{\cdot}001$.

The increased prevalence of cytotoxic sera in lepromatous patients with HBsAg may well be due to a postulated higher titre of HBsAg in these patients than in controls. A higher rate of HBsAg in lepromatous patients has been reported previously (Blumberg *et al.*, 1967) probably due to their generalized poor immune response. Lepromatous patients may well attain a higher titre of HBsAg before stimulation of production of anti-HBs to terminate the HBV infection. This hypothesis can be tested in this Papua New Guinean data set by examining lymphocytotoxicity in those with detectable HBsAg by the relatively insensitive assay of immunoelectrophoresis (IEOP). Only those sera with the highest HBsAg titres will be recognized by IEOP as HBsAg positive and, under the hypothesis of HBsAg titre-related cytotoxicity, they should be the most strongly lymphocytotoxic. In fact, IEOP recognized only seventeen persons as positive for HBsAg, although forty-eight were positive by haemagglutination test. Of those sera IEOP positive, 41% were highly cytotoxic ($\bar{X}>1{\cdot}0$) compared with 14% of IEOP negative sera

TABLE 4. Frequency distributions of cytotoxicity scores

HBV status		Number	Lymphocytotoxicity score (%)					Cytotoxic score
HBsAg	anti-HBs	tested	0–0·2	0·2–0·4	0·4–0·8	0·8–1·6	1·6+	>0·8 (%)
Lepromatous								
+	−	15	13	7	13	47	20	67
−	+	20	30	40	20	10	0	10
−	−	33	24	24	39	12	0	12
Non-lepromatous								
+	−	27	19	26	30	22	4	26
−	+	31	7	19	29	39	7	46
−	−	33	30	15	15	39	0	39

($\chi_1^2 = 8{\cdot}15$, $P<0{\cdot}01$), partly accounting for the higher rate of lymphocytotoxicity in leprosy patients but also reconfirming the significance of HBsAg as a cytotoxic determinant.

An alternative explanation for the lepromatous leprosy and HBsAg interaction in producing LCAs may be that since HBsAg is found more frequently in institutionalized lepromatous patients than in out-patients (Serjeantson & Woodfield, 1978), HBsAg is simply a marker defining a subgroup of patients with lepra reactions and a heavier drug regime. However, there was no correlation between hospitalization and LCAs. In thirty-two lepromatous patients with a history of hospitalization, mean cytotoxicity was $0{\cdot}58 \pm 0{\cdot}08$ compared with $0{\cdot}51 \pm 0{\cdot}07$ in thirty-nine lepromatous out-patients.

DISCUSSION

Our results show no increased prevalence of LCAs in healthy controls and tuberculoid patients with asymptomatic HBsAg. This finding is in agreement with DeHoratius *et al.* (1976), who did not detect lymphocytotoxins in ten chronic carriers of HBsAg although LCAs were present in serum of patients with chronic and acute viral hepatitis. The absence of lymphocytotoxins in healthy persons with HBsAg suggests that the DNA-binding antibodies reported in this group (Kingham *et al.*, 1978) are not due necessarily to release of intracellular contents of lymphocytes into the circulation, as may be the case in SLE (Ooi *et al.*, 1974), but could well be induced by viral DNA as proposed by Kingham *et al.* (1978).

Lepromatous leprosy patients, in contrast with the control group, had significantly elevated LCAs in the presence of HBsAg. This disease-associated lymphocytotoxicity may be explained in part, though somewhat inadequately, by postulated higher titres of HBsAg in lepromatous leprosy. Certainly, in this study the sera IEOP-positive for HBsAg were significantly more cytotoxic than sera with HBsAg detectable only by the more sensitive haemagglutination test, but even within the small group of IEOP-positive persons the HBsAg and lepromatous leprosy interaction was apparent.

Although HBsAg is a poor indicator of *in vitro* lymphocytotoxicity, when it occurs in conjunction with certain diseases such as lepromatous leprosy or chronic active hepatitis, lymphocytotoxicity is enhanced. This could be coincidental to the presence of HBsAg, which may simply reflect an impaired immune response in the host. Alternatively, it may reflect an abnormal response to HBV. Cold-reacting lymphocytotoxic antibodies are maintained in the serum for several weeks only following acute viral infection (Huang *et al.*, 1973) or vaccination (Kreisler *et al.*, 1970) suggesting that continued exposure to the infecting agent is necessary for maintenance of detectable LCAs. Continued exposure to HBV can be expected in the study population where the virus is hyperendemic. However, in healthy controls and tuberculoid patients, persistence of viral coat-protein in the serum may not be indicative of repeated HBV infection as could well be the case in lepromatous patients with their generalized impaired immune response.

One explanation for the occurrence of LCAs is that the IgM antibodies recognize a complex of antigen and lymphocyte receptors and then cross-react with receptors alone on normal lymphocyte membranes. This hypothesis gains support from investigations in laboratory animals, where ability to produce anti-idiotypic antibodies has been demonstrated (Iverson, 1970). Nisonoff & Bangasser (1975) have shown that anti-idiotypic antibody, reactive with determinants within or near a specified antigen-binding site, can have anti-lymphocyte receptor activity. If lepromatous patients with HBsAg have not only circulating viral coat-protein but also continued HBV infection, this may explain the interaction between HBsAg and lepromatous leprosy in production of LCAs. The particular susceptibility of lepromatous patients to HBV is well-documented (Blumberg *et al.*, 1967; Serjeantson & Woodfield, 1978).

Lepromatous patients without HBsAg have a significantly depressed level of LCAs suggesting that non-specific disease involvement of vital organs is not responsible for LCA production in the HBsAg positive group. LCAs were depressed markedly in lepromatous patients with either anti-HBs or with no serological evidence of exposure to the virus, when compared with controls who, in this population, had a high background level of LCAs. The depressed prevalence of LCAs in lepromatous patients without HBsAg may be related to their immune deficiency of delayed-type hypersensitivity. If LCAs are a feedback mechanism stimulated by cell-mediated immunity, then lepromatous patients could be expected

to have depressed LCA production. Serum inhibitory factors, as yet unidentified but possibly LCAs, are detectable in chronic and acute hepatitis patients at the time when antibody-dependent cellular cytotoxicity is reduced (Bortolotti, Realdi & Fattovich, 1978).

It is possible that LCAs in lepromatous patients with HBsAg are coincidental to the presence of HBsAg itself, which may simply indicate an unusual subgroup within lepromatous leprosy. However, we are unable to identify such a subgroup. The majority of patients with a history of lepra reactions had a history of recurrent hospitalization, yet hospitalization was not associated with either LCAs or with the HBsAg and lepromatous leprosy interaction in production of LCAs. Similarly, immunosuppressive therapy has not been related to LCAs in any disease yet examined (Strickland *et al.*, 1975; Winfield *et al.*, 1975). Finally, immune complexes, known to be efficient stimulators of LCA production in experimental animals (McKearn, Stuart & Fitch, 1974) are unlikely to be confined to the group of lepromatous patients with HBsAg. The majority of both lepromatous and tuberculoid leprosy patients have circulating immune complexes (Bjorvatn *et al.*, 1976).

Our results may explain the equivocal findings of various reports investigating autoantibodies in leprosy. Bonomo & Dommacco (cited in Petchclai *et al.*, 1973) and Matthews & Trautman (1965) found high prevalences of rheumatoid factor in lepromatous leprosy whereas Petchclai *et al.* (1973), studying a different population, found no marked increase in autoantibodies in leprosy. These authors suspected a difference in immune response among Thais accounted for by the absence of autoantibodies. Since rheumatoid factor and anti-nuclear factor are correlated positively with LCAs in autoimmune diseases (Terasaki *et al.*, 1970), our finding of two subgroups within lepromatous patients, one with elevated and the other with depressed LCAs, is in accord with the hypothesis of differing host immune responses within lepromatous leprosy.

This conclusion is further supported by the findings of McAdam, Fudenberg & Michaeli (1978) who also examined leprosy patients from Papua New Guinea. In a Papua New Guinean highland population, anti-collagen autoantibodies were elevated markedly in lepromatous patients but not in tuberculoid patients. In contrast, in a coastal population, anti-collagen antibodies were elevated to the same degree in all forms of leprosy. McAdam *et al.* (1978) suggest that the many intercurrent infections, apart from leprosy, in a tropical coastal area obscure the relationship between lepromatous leprosy and serum autoantibodies. Our results suggest that an important superimposed infection which interacts with lepromatous leprosy in production of autoantibodies is that of hepatitis B virus.

The authors wish to thank staff members of the Hatzfeldhaven Health Centre, Papua New Guinea, for assistance during data collection, Dr D. G. Woodfield for HBV assays, Dr R. Anders for helpful comments on the manuscript and Mrs R. Williams for clerical assistance.

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