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Immunogenetic analysis of patients with post-schistosomal liver cirrhosis in man

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SUMMARY

Immune responsiveness of 121 patients with post-schistosomal liver cirrhosis to schistosomal antigens was investigated. Out of 78 patients, only five (6·4%) showed low responsiveness to schistosomal adult worm antigen whereas 73 (93·6%) were high responders. Out of 57 healthy individuals with previous schistosomal infection, low responders were found in 17·5%. The frequency of low responders to schistosomal adult worm antigen was significantly decreased in the patients with post-schistosomal liver cirrhosis ($P < 0·05$). Out of 121 patients, a significant increase in frequency of HLA-Bw44-DEn haplotype was observed (corrected $P < 0·02$). On the other hand, HLA-Bw52-Dw12 haplotype which was reported to be in strong linkage disequilibrium with an immune suppression gene for schistosomal adult worm antigen was significantly decreased (corrected $P < 0·005$). These observations suggested that an HLA-linked immune suppression gene controlled susceptibility or resistance to post-schistosomal liver cirrhosis through regulation of immune responsiveness of the hosts to schistosomal antigen in man.

INTRODUCTION

Hepatosplenic schistosomiasis in experimental animals was caused by granulomatous response of the hosts to schistosomal antigens (Warren, 1972), and this granulomatous response reflected the host's immune response to the schistosomal antigens (Buchanan, Fine & Colley, 1973; Warren, 1975; Colley, 1976). In human schistosomiasis, only a proportion of individuals develop post-schistosomal liver cirrhosis (PSLC) (Inaba *et al.*, 1977). This observation suggested the existence of host factors in development of PSLC as well as parasitic factors.

There is a series of reports that shows strong associations between HLA and immune responsiveness in man (Greenberg, Gray & Yunis, 1975; Spencer, Cherry & Terasaki, 1976; de Vries *et al.*, 1977; Sasazuki *et al.*, 1978, 1980c; Nose *et al.*, 1980). Since HLA is comparable to the murine H-2 complex in which immune response genes (Ir genes) and immune suppression genes (Is genes) are mapped (Benacerraf & McDevitt, 1972; Benacerraf & Dorf, 1976), Ir genes and Is genes in man have been assumed to be located in the HLA region. Indeed Sasazuki *et al.* (1980b) clearly demonstrated an HLA-linked immune suppression gene for streptococcal antigen in man. We also reported the strong association between HLA-Bw52-Dw12 haplotype and low responsiveness to schistosomal adult worm antigen (Sasazuki *et al.*, 1980c). This strong association indicated the existence of an HLA-linked Is gene controlling low responsiveness to schistosomal adult worm

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antigen. The diversity in immune responsiveness to schistosomal antigen in man might contribute to the susceptibility or resistance to hepatosplenic schistosomiasis.

In this paper we present the immunogenetic features of the patients with PSLC and discuss the possible mechanism for developing PSLC in man.

PATIENTS AND METHODS

Patients. One hundred and twenty-one unrelated patients with PSLC were randomly picked at the outpatient clinic of Kofu City Hospital. Out of the 121 patients, 85 were males and 36 females. The age distribution ranged from 39 to 80 years. Diagnosis of PSLC was based on the finding of a liver biopsy that showed portal cirrhosis with densely scattered schistosomal eggs. Previous schistosomal infection was documented by skin test, circum oval precipitin test or the existence of eggs in the liver or rectal biopsy specimens. Two groups were studied as controls; 19 patients with liver cirrhosis without previous schistosomal infection and 57 healthy individuals with previous schistosomal infection.

Lymphocyte preparation. Peripheral blood was obtained from each patient with heparin as the anticoagulant (100 units/ml). The blood was mixed with the same volume of phosphate-buffered saline (pH 7·2), and the mixture was layered on Ficoll-Conray gradient solution (Sp. gr. 1·077). After centrifugation at 1,350 rpm for 35 min, lymphocytes were collected and washed once with Hank's solution (Nissui Seiyaku Co., Tokyo).

HLA-A,-B,-C typing. HLA-A,-B,-C typing was performed by the method of NIH standard microcytotoxicity test (Manual of tissue typing technique, 1976). The typing reagents used in this study were 109 sera for 14 HLA-A specificities, 22 HLA-B specificities and six HLA-C specificities.

HLA-D typing. HLA-D typing was performed by mixed lymphocyte culture (MLC) test using seven homozygous typing cells for Dw1, Dw2, Dw3, Dw4, Dw12, DYT and DEn. Typing cells were treated with 100 μg of mitomycin C (Kyowa Hakko Co., Tokyo) and washed with RPMI 1640 (Nissui Seiyaku Co., Tokyo) three times. Typing cells were then suspended in RPMI 1640 (GIBCO., Grand Island, New York, USA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 20% heat-inactivated pooled male sera. Cultures were set up in triplicate in round bottomed microtitre plates (Nunc, Rockilde, Denmark). Fifty thousand responder cells and the same number of typing cells were mixed in 0·2 ml of the medium in a humidified atmosphere, 5% CO_2 and 95% air for 6 days. One microcurrie of ^{3}H-thymidine was added into each well, and cells were harvested after 16 hr. Incorporation of ^{3}H-thymidine into responder cells was counted in a liquid scintillation counter.

Preparation of schistosomal antigens. Schistosomal adult worm antigen (Sj VBS antigen) was prepared from adult *Schistosoma japonicum* by the method described by Chaffee, Bauman & Shapilo (1954). Schistosomal egg antigen (SEA) was prepared by the method described by Boros & Warren (1970). Protein content was measured by Lowry's method (Lowry *et al.*, 1951). The antigens were sterilized by passing through a millipore filter membrane (pore size 0·22 μm) (Millipore Corp., Bedford, Massachusetts, USA) and stored at −80°C.

Immune response to schistosomal antigens in vitro. Twenty thousand peripheral lymphocytes were cultured in flat bottomed microtitre plates (Nunc) with 0·2 ml of fully supplemented medium containing 10% heat-inactivated human pooled male sera. One microgram of Sj VBS antigen or SEA was added and the plates were incubated in a humidified atmosphere, 5% CO_2 and 95% air at 37°C for 6 days. After 6 days incubation, 1 μCi of ^{3}H-thymidine was added into each well, and the cells were harvested after 16 hr incubation. Incorporation of ^{3}H-thymidine was counted in a liquid scintillation counter. Data analysis was performed using lnΔc.p.m. calculated as follows,

$$\ln\Delta\text{c.p.m.} = \ln(\text{test c.p.m.} - \text{negative control c.p.m.})$$

RESULTS

Immune response to Sj VBS antigen in vitro

Immune response of the patients with PSLC to Sj VBS antigen *in vitro* ranged widely from lnΔc.p.m.

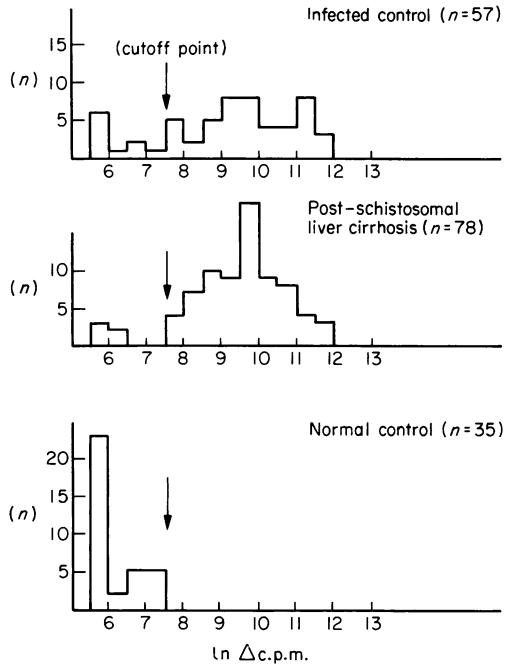

Fig. 1. Immune responsiveness to schistosomal adult worm antigen of healthy infected control, patients with PSLC and normal control. Immune responsiveness was measured by antigen specific proliferative response of the peripheral T lymphocytes *in vitro*. Since normal control did not show responsiveness stronger than lnΔc.p.m. = 7·5, we arbitrarily decided lnΔc.p.m. = 7·5 as cutoff point for high and low responsiveness. Significant decrease in frequency of low responders was observed in the patients with post schistosomal liver cirrhosis compared with healthy infected control.

6·0 to 11·8 (Fig. 1). We arbitrarily decided lnΔc.p.m. = 7·5 as a cutoff point to divide the patients into low and high responders, based on the fact that normal control did not show responsiveness stronger than lnΔc.p.m. = 7·5. Out of 78 patients, only five (6·4%) were assigned as low responders and 73 (93·6%) as high responders. Out of 57 healthy individuals with previous schistosomal infection, 10 (17·5%) were assigned as low responders and 47 (82·5%) as high responders. The frequency of low responders in the patients with PSLC was significantly lower than the infected control group without PSLC ($P < 0·05$).

Immune response to SEA in vitro

A histogram of immune response of the patients to SEA was shown in Fig. 2. The distribution of the response showed clear bimodality, and lnΔc.p.m. = 7·5 was decided as a cutoff point for high and low responsiveness. Out of 74 patients 40 (54·1%) were assigned as high responders and 34 (45·9%) as low responders.

As controls, we studied immune responses of the patients to streptococcal cell wall antigen or phytohaemagglutinin *in vitro*. Between the patient group and the normal healthy controls, we did not observe any significant difference in responsiveness to the antigen or mitogen (Fig. 3).

HLA specificities in the patients with PSLC

The frequencies of HLA-Bw44 and DEn were increased to 28·9% and 36·2% in the patients with PSLC compared with 10·0% and 17·2% respectively in the healthy Japanese controls (relative risk = 3·66, corrected $P < 0·0005$ for Bw44, relative risk = 2·73, corrected $P < 0·01$ for DEn). Subsequently the HLA-Bw44-DEn haplotype, which is one of the commonest HLA-B-D

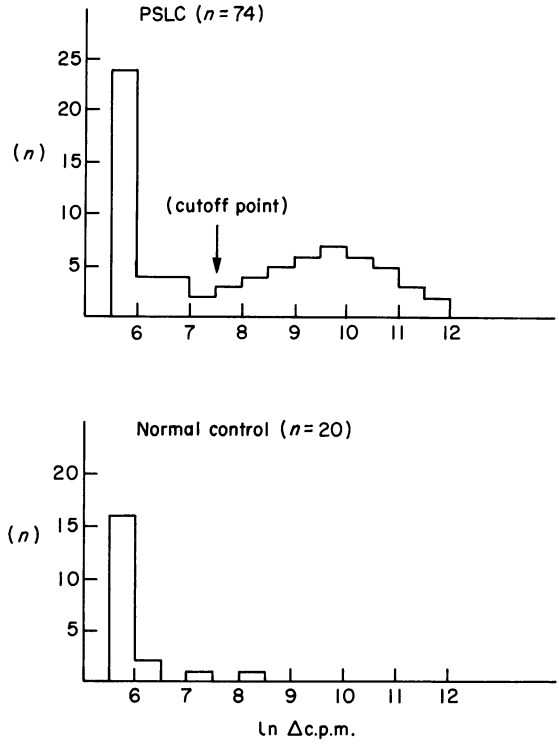

Fig. 2. Immune responsiveness to soluble egg antigen (SEA) of patients with PSLC and normal control. Almost the same number of high responders and low responders to SEA was observed in the patients with PSLC.

haplotypes in the Japanese population, was significantly increased in the patient group (haplotype frequency = 0·159, relative risk = 2·96, corrected $P < 0·02$). On the other hand, HLA-Bw52 and Dw12 were significantly decreased in frequency to 5·1% and 12·7% in the patients compared with 20·5% and 27·4% respectively in the healthy Japanese controls (relative risk = 0·208, corrected $P < 0·005$ for Bw52; relative risk = 0·384, corrected $P < 0·05$ for Dw12). Moreover, only two patients with PSLC had HLA-Bw52-Dw12 haplotype (haplotype frequency = 0·0127) whereas this HLA

Table 1. Association between HLA and patients with post-schistosomal liver cirrhosis

HLA	Sj(+)LC ($n=121$)	Sj(−)LC ($n=19$)	Normal control ($n=220$)	Relative risk*	*P*corr* <
HLA-Bw44	28·9%	10·5%	10·0%	3·66	0·0005
HLA-Bw52	5·1%	21·1%	20·5%	0·208	0·005
HLA-DEn	36·2%	NT	17·2%	2·73	0·01
HLA-Dw12	12·7%	NT	27·4%	0·384	0·05
HLA-Bw44-DEn	0·159†	NT	0·063†	2·96	0·02
HLA-Bw52-Dw12	0·0127†	NT	0·097†	0·113	0·005

* Sj(+)LC vs normal control; † haplotype frequency.

Sj(+)LC = patients with post-schistosomal liver cirrhosis; Sj(−)LC = patients with liver cirrhosis without schistosomal infection; *P*corr = corrected *P*.

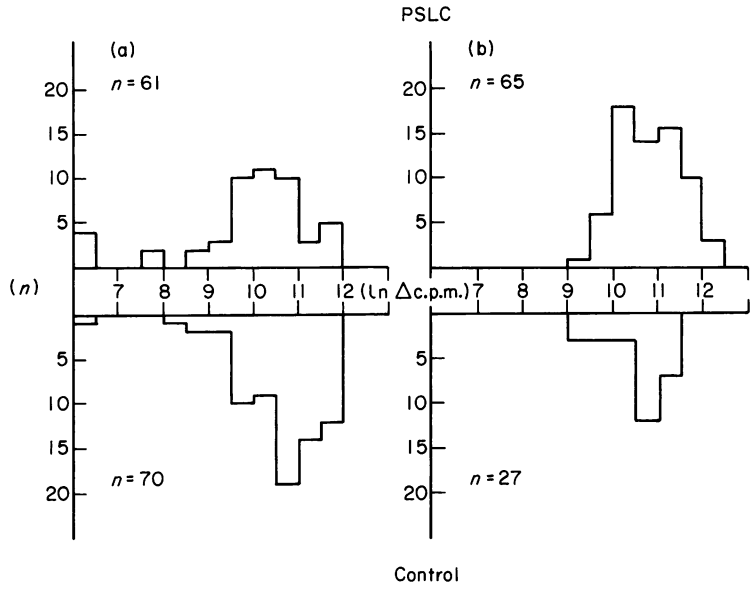

Fig. 3. *In vitro* proliferative response to non-schistosomal antigen or mitogen of patients with PSLC and normal control. When streptococcal cell wall antigen (a) and phytohaemagglutinin (b) were used as non-schistosomal antigen or mitogen, there was no significant differences in responsiveness between these two groups.

haplotype was observed in high frequency (haplotype frequency = 0·0953) in the Japanese population (Sasazuki *et al.*, 1980a) (relative risk = 0·113, corrected $P < 0·005$) (Table 1). The patients with liver cirrhosis without schistosomal infection showed no significant distortion in frequency of these HLA specificities compared with the healthy Japanese controls (Table 1).

DISCUSSION

Although the close relation between schistosomal infection and development of hepatosplenic lesion has been epidemiologically documented in man, the biological mechanisms involved in human schistosomiasis are not fully understood. Recently, Deelder *et al.* (1980) reported that there was a big difference in susceptibility to hepatosplenic schistosomiasis among different strains of mice and that there was a strong association between immune response to schistosomal antigen and susceptibility to hepatosplenic schistosomiasis. By utilizing two H-2 congenic strain mice, C3H.B10 (H-2^b) and C3H/Sn (H-2^k), they showed that an H-2 linked Ir gene(s) or Is gene(s) governed the susceptibility to hepatosplenic schistosomiasis through the control of immune response of the hosts to schistosomal antigens.

In human schistosomiasis we have already shown that there was a strong association between low responders to schistosomal antigen and HLA-Bw52-Dw12 haplotype (Sasazuki *et al.*, 1980c). Several groups have also observed the diversity in humoral and cellular immune responsiveness in human schistosomiasis (Warren, Kellermeyer & Jordan, 1973; Perry, Warren & Jordan, 1977; Colley *et al.*, 1977a; Ellner *et al.*, 1980b). This diversity might account for the varied susceptibility to hepatosplenic schistosomiasis similar to that observed in murine schistosomiasis.

We found that the 93·6% of the patients with PSLC responded vigorously to Sj VBS antigen, and that the frequency of HLA-Bw52-Dw12 haplotype which was suggested to be in linkage disequilibrium with an Is gene for Sj VBS antigen was decreased in the patient group. These findings seemed to indicate that high immune responsiveness to Sj VBS antigen contributed to developing

PSLC whereas low responsiveness to this antigen prevented the infected individuals from developing PSLC. This concept was not inconsistent with the report by Ellner *et al.* (1980b) that the patients with hepatosplenic schistosomiasis showed vigorous response to adult worm antigen compared with non-hepatosplenic group. In the healthy inhabitants with previous schistosomal infection, our intensive investigation revealed that five of 15 high responders (33·3%) had some liver involvement such as jaundice, hepatomegaly and/or ascites in their past history whereas none of 10 low responders had liver involvement in their past history (Ohta, unpublished data).

Since HLA-Bw44-DEn haplotype was not associated with either liver cirrhosis without schistosomal infection or the healthy infected individuals, the association of PSLC with HLA-Bw44-DEn haplotype was specific for the patients with PSLC. An HLA-linked Ir gene for Sj VBS antigen might explain the association between HLA-Bw44-DEn haplotype and PSLC. If so, in the healthy population with previous schistosomal infection HLA-Bw44-DEn haplotype should show a statistical association with high responsiveness to Sj VBS antigen. Nine out of 11 individuals with HLA-Bw44 were high responders and two individuals with Bw44 who showed low responsiveness to Sj VBS antigen carried HLA-Bw52-Dw12 haplotype which was assumed to carry an Is gene for Sj VBS antigen. A strong association between hepatosplenic schistosomiasis and HLA-A1 and -B5 in the Egyptian population has also been reported (Salam, Ishaac & Mahmoud, 1979).

In the analogy of the murine schistosomiasis, the immune response of the host to schistosomal egg antigen was expected to have a crucial role in human hepatosplenic schistosomiasis (Warren, 1972). However, we observed that there were the same number of high and low responders to SEA in the patients with PSLC, and we could not identify a significant role of the immune response to SEA in the pathogenesis of PSLC. Ellner *et al.* (1980b) also could not elucidate the role of immune response to SEA in development of hepatosplenic schistosomiasis in the Egyptian population. It has been reported that immune responses to SEA were diminished in the early infection phase in human schistosomiasis by suppressor factor and/or suppressor cells (Colley *et al.*, 1977a, 1977b; Ottesen *et al.*, 1978; Ellner *et al.*, 1980a; Rocklin, Tracy & Kholy, 1981). Therefore, there are two alternatives to explain our observations: (1) although a vigorous response to SEA had a crucial role in development of PSLC, this strong response was already suppressed by immunoregulatory system in our chronic patients or (2) in human schistosomiasis the immune response to SEA did not have any crucial role in development of PSLC. A recent study of Doughty & Phillips (1982) showed that a typical granulomatous response was observed only when the host was exposed to both viable adult worms and eggs. This fact strongly suggested that an immune response to egg alone could not cause the hepatosplenic lesion, and that the immune response to adult worm has a crucial role in development of the symptoms.

In summary, most patients with PSLC responded vigorously to Sj VBS antigen *in vitro*. The number of low responders in the patients with PSLC was significantly smaller than in the schistosomal infected individuals without PSLC. Only two patients with PSLC had HLA-Bw52-Dw12 haplotype which was suggested to be in strong linkage disequilibrium with an Is gene for Sj VBS antigen. These results suggested that high immune responses to Sj VBS antigen might cause the development of PSLC whereas low responses to Sj VBS antigen, governed by an Is gene in strong linkage disequilibrium with HLA-Bw52-Dw12 haplotype, would lead to resistance to PSLC. The patients with PSLC showed a strong association with HLA-Bw44-DEn haplotype suggesting the existence of an Ir gene for Sj VBS antigen in linkage disequilibrium with this HLA haplotype.

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