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Mechanism of a lymphocyte abnormality associated with HLA-B8/DR3: role of interleukin-1

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SUMMARY

Lymphocytes from normal individuals with the histocompatibility antigens HLA-B8 and DR3 have impaired proliferative responses when stimulated with suboptimal concentrations of mitogens. We have previously shown that an important factor in the impaired response is a failure to produce normal quantities of interleukin-2 (IL-2). To examine the mechanism of decreased responsiveness further, we measured interleukin-1 (IL-1) production of low responder subjects compared with controls. The peripheral blood mononuclear cells of five low responder individuals with HLA-B8/DR3 stimulated with 0·05 μg/ml of phytohaemagglutinin (PHA) accumulated only 0·036 U/ml of IL-1 compared with 0·32 U/ml for normal responders. There was a highly significant correlation between the PHA-stimulated IL-1 concentration at 12 h and the subsequent IL-2 concentration at 48 h ($r=0·89$, $P<0·0001$) suggesting a role of decreased IL-1 production in the impaired response. A study of unfractionated or column-fractionated culture supernatants revealed no evidence that the decreased IL-1 activity in the supernatants of low responder subjects was related to increased IL-1 inhibitor concentrations. These results suggest that impaired IL-2 production and lymphocyte proliferation in healthy subjects with HLA-B8/DR3 may be mediated at least in part by decreased IL-1 production, and implicates a defect of a very early event in lymphocyte activation.

Keywords HLA-B8/DR3 interleukin-1 lymphocyte activation cytokines

INTRODUCTION

The histocompatibility antigens HLA-B8 and DR3 are associated with at least nine immune-mediated diseases including Sjögren's syndrome, myasthenia gravis, Graves' disease and coeliac disease (Schwartz & Shreffler, 1980). Despite numerous disease associations, the two antigens (which are frequently inherited together as a haplotype) are quite common, especially in Northwestern Europe, with a prevalence approaching 50% in the West of Ireland (McKenna *et al.*, 1983). It has been suggested that the genes encoding HLA-B8/DR3 may have become frequent by conferring increased resistance to infectious disease (van Rood, van Hooff & Keuning, 1975). Indeed, it seems plausible that a genetically determined 'hyper-responsiveness' could provide an individual with augmented resistance to infection and at the same time increase the risk of autoimmunity.

Several investigators including ourselves have studied immunological parameters in normal subjects with HLA-B8/DR3 to determine whether measureable differences in immune responsiveness could support a hypothesis that individuals with these antigens are different in some way. Several special immunological characteristics have been found to be associated with HLA-B8/DR3, including decreased responsiveness to T cell mitogens (Lawley *et al.*, 1981; McCombs & Michalski, 1982; Amer *et al.*, 1986) increased numbers of immunoglobulin producing cells after stimulation with pokeweed mitogen (Ambinder *et al.*, 1982; McCombs *et al.*, 1986), and an increased ratio of CD4-to-CD8 bearing T cells (McCombs *et al.*, 1986).

One of the most striking of these immunological 'abnormalities' of subjects with HLA-B8/DR3 is an impaired response to suboptimal concentrations of phytohaemagglutinin (PHA) and concanavalin A (McCombs & Michalski, 1982). We have previously shown that the impaired proliferative response is associated with and probably due, in large part, to impaired production of interleukin-2 (IL-2) (Hashimoto, McCombs & Michalski, 1989). The purpose of the present study was to determine whether the impaired production of IL-2 reflects at least in part an even earlier abnormality of the events leading to lymphocyte activation: decreased production of IL-1, a major regulatory molecule for the optimal production of IL-2.

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MATERIALS AND METHODS

Subjects

The subjects were all healthy students and medical centre personnel aged 22–48 years. At least one normally responding HLA-B8/DR-negative subject was paired with and studied on the same occasion as the HLA/DR3-positive individuals.

Mitogens and lymphokines

Purified PHA was obtained from Burroughs Wellcome (Research Triangle, NC), silica from Nippon Kohtaiken Kyusho (Takasahi, Japan), lipopolysaccharide (LPS) from Difco Laboratories (Detroit, MI), and recombinant IL-2 from Amgen Biologicals (Thousand Oaks, CA). IL-1 was prepared by stimulating purified human monocytes with silica particles for 12 h. This preparation had no significant IL-2 activity when tested in a CTLL microassay (Gillis *et al.*, 1978).

HLA A, B, C and DR typing

HLA antigens of the A, B, C, and DR loci were detected by a microcytotoxicity method (Terasaki *et al.*, 1978) using trays purchased from Lambda One (Los Angeles, CA). For HLA-DR typing, B lymphocytes were recovered from soda-straw nylon wool columns (Danilovs, Auayoub & Terasaki, 1980).

Cell preparation

Peripheral blood mononuclear cells PBMC were separated from heparinized venous blood on Ficoll–Hypaque gradients (s.g. 1·077). Purified monocytes were prepared by suspending PBMC in RPMI–1640 supplemented with 10% fetal calf serum (FCS) at 1×10 cells/ml. One millilitre was added to each well of a 24-well plate (Costar, #34240, Cambridge, MA) and incubated at 37°C, in 5% CO_2 in air in a humidified atmosphere for 2 h. The non-adherent cells were removed and the monocyte layers washed three times. The monolayers were used in these experiments by adding medium and silica or LPS directly to the wells.

Lymphocyte proliferation assay

PBMC were cultured in flat-bottomed microplates (2×10^5 cells/well) in 0·2 ml of culture medium. To measure proliferation, the cells were stimulated with various concentrations of PHA in quadruplicate cultures and incubated at 37°C in an humidified 5% CO_2 atmosphere for 68 h followed by a 4-h pulse with ^{3}H-thymidine (1 μCi/well; specific activity 6·7 Ci/mmol; ICN, Irvine, CA). The cells were harvested onto glass fiber strips with a cell harvester (Otto Hiller, Madison, WS), and retained radioactivity was counted in a scintillation counter.

IL-2 assay

To measure IL-2 accumulation, PBMC (2×10^5 cells/well) were stimulated for 48 h. The cultures were harvested, centrifuged and the supernatants collected and stored at −70°C. IL-2 activity was assayed as described by Gillis *et al.* (1978). Murine IL-2 dependent cytotoxic T lymphocyte (CTLL-2) indicator cells were suspended at 5×10^4 cells/ml in HEPES-buffered RPMI-1640 supplemented with 10% heat-inactivated FCS, 1% antibiotic–antimycotic, 1% glutamine and 5×10^{-5} M 2-mercaptoethanol. One tenth of a milliliter (5×10^3 cells) was placed in each microplate well with 0·1 ml of two-fold dilutions of culture supernatant from PHA-stimulated PBMC or a standard IL-2 preparation. The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 20 h, and followed by a 4-h pulse with ^{3}H-thymidine (0·5 μCi/well; specific activity 25–30 Ci/mmol) before harvest and counting.

Calculation of IL-2 activity

IL-2 activity was calculated by a modification of probit analysis (Gillis *et al.*, 1978) using serial two-fold dilutions of a standard IL-2 preparation of our own as a reference. A dose-response curve was constructed and the maximum amount of thymidine incorporation was designated 100%. Thymidine incorporation induced by dilutions of the samples and standard were then converted to a percent of maximum incorporation and plotted against that dilution on log/log graph paper. The best fitting straight line was determined by regression analysis using the applied statistics program of a Texas Instruments (Lubbock, TX) calculator and U/ml were calculated using equations previously described (Hashimoto, McCombs & Michalski, 1989)

IL-1 assay

The IL-1 concentration in the supernatants of PBMC and monocyte cultures stimulated for 12 h was determined by a mouse thymocyte co-stimulation assay as previously described (Mizel, 1980). Briefly, single cell suspensions of mouse thymocytes from 5- to 8-week-old C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME), were prepared by teasing thymus tissue through a small sieve. The cells were suspended in tissue culture medium supplemented with 10% FCS and $2{\cdot}5 \times 10^{-5}$ M 2-mercaptoethanol at $1{\cdot}5 \times 10^6$/0·2 ml per well with 0·5 μg/well PHA and either serial dilutions of the supernatants to be tested, or of an IL-1 standard. The cultures were incubated for 72 h, and proliferation was determined by ^{3}H-thymidine incorporation.

Calculation of IL-1 activity

IL-1 activity was calculated by a modification of probit analysis (Gillis *et al.*, 1978) using a single standard IL-1 preparation as a reference. Serial two-fold dilutions of the standard were used to construct a dose-response curve and the maximum amount of thymidine incorporation was designated 100%. Thymidine incorporation induced by dilutions of the samples and standard were then converted to a percent of maximum incorporation and plotted against that dilution on log/log graph paper. The best fitting straight line was determined by regression analysis and the dilution that stimulated 50% of maximum incorporation was calculated. Our standard IL-1 preparation had 54 half-maximal U/ml. Samples were compared with the 50% line of maximum incorporation induced by this standard using the following equation:

$$\text{IL-1 (U/ml)} = \frac{\text{Reciprocal of titre of the } \textit{sample} \text{ giving 50\% max ct/min}}{\text{Reciprocal of titre of the } \textit{standard} \text{ giving 50\% max ct/min}} \times 54 \text{ U}$$

The data presented is the net IL-1 secretion after subtraction of the unstimulated (medium) control.

IL-1 inhibitor assay

Column-fractionated supernatants from PHA-stimulated PBMC were assayed for relative IL-1 inhibitor activity as

Table 1. Proliferation of lymphocytes and lymphokine production by PBMC in response to PHA, and IL-1 production by monocytes in response to LPS and silica

	HLA-B8/DR3+	HLA-B8/DR3−	*P*
PBMC response to PHA (0·05 μg/ml)			
IL-1 concentration at 12 h	0·036*	0·32	<0·001
	−1·44±0·19†	−0·50±0·12	
IL-2 concentration at 48 h	0·06	1·67	<0·001
	−1·22±0·23	0·22±0·07	
Proliferative response	328‡	5782	<0·003
	2·52±0·25§	3·77±0·08	
Monocyte response to LPS IL-1 production	14·8*	15·9	NS
	1·17±0·06†	1·20±0·10	
Monocyte response to silica IL-1 production	40·4	29·4	NS
	1·61±0·11	1·47±0·08	

PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; IL, interleukin; LPS, lipopolysaccharide; NS, not significant.

*Geometric net mean U/ml of lymphokine.

†U/ml of IL-1: log 10 (mean±s.e.m.).

‡Geometric mean net ct/min.

§ct/min: $\log_{10}$ (mean±s.e.m.).

previously described (Berman *et al.*, 1986). Graphs of inhibitor activity were plotted on paper and the area of inhibition was cut out and weighed as a measure of total inhibitor activity of a sample.

Statistical analysis

Differences between the means of log transformed proliferative and lymphokine data were tested by Student's *t*-test. Correlations of log transformed data were made by linear regression analysis. All statistical calculations were made using a software module provided for the TI-59 programmable calculator (Texas Instruments).

RESULTS

Impaired proliferative responses to suboptimal doses of PHA are associated with decreased IL-1 concentrations in culture supernatants

PBMC were cultured with 0·05 μg/ml of PHA and supernatants were harvested at 12 and 48 h, respectively, for measurement of IL-1 and IL-2. Proliferative responses were determined at 72 h. The mean concentration of IL-1 in mononuclear cell supernatants of five low responder subjects with HLA-B8/DR3 was 0·036 U/ml compared with 0·32 U/ml for six normal responders ($P<0·001$, Table 1). The magnitude of the reduced IL-1 concentration was comparable to the reduction in IL-2 concentration and proliferative response of these same subjects.

In contrast, purified monocytes from these individuals, cultured with either LPS or silica, produced IL-1 concentrations with no significant differences between the two groups (Table 1).

Low concentrations of IL-1 at 12 h of culture are associated with decreased production of IL-2 over the next 36 h

We have previously shown with the use of antibodies to the IL-2 receptor that the decreased IL-2 concentrations in subjects with HLA-B8/DR3 are secondary in large part to decreased IL-2 production rather than increased utilization (Hashimoto *et al.*, 1989). To determine whether a low IL-1 concentration accumulated by an individual's mononuclear cells after 12 h of stimulation with PHA is associated with decreased IL-2 production at 48 h, we used regression analysis to compare the levels of the two lymphokines in 13 subjects. There was a highly significant correlation between an individual's IL-1 concentration at 12 h and the subsequent accumulation of IL-2 ($r=0·89$, $P<0·001$; Fig. 1a). As previously shown in a larger group of subjects, the IL-2 concentrations at 48 h produced by the lymphocytes of these individuals correlated with the proliferative response at 72 h ($r=0·93$, $P<0·001$; Fig. 1b).

Impaired production of IL-1 by PHA-stimulated mononuclear cells does not reflect a decreased ability of the monocytes to produce IL-1 when directly stimulated by LPS or silica

In order to determine whether a low concentration of IL-1 after stimulation of mononuclear cells with a suboptimal concentration of PHA reflected a qualitative abnormality of monocyte function, we measured IL-1 production directly stimulated by culturing purified monocytes with LPS or silica. We then used regression analysis to compare each individual's PHA-induced mononuclear cell IL-1 concentration with that produced by purified monocytes stimulated with LPS or silica. There was no correlation between the levels of IL-1 stimulated by PHA and LPS (Fig. 2a) and low levels of PHA-stimulated IL-1 were actually associated with somewhat higher silica reactivity ($r=0·49$, $P=0·062$; Fig. 2b).

IL-1 activity levels measured by the mouse thymocyte activity are not affected by IL-2

Although the 12 h supernatants that we used for our IL-1 studies had no detectable IL-2 by the CTLL-2 microassay, it seemed prudent to determine whether mouse thymocytes may respond to levels of IL-2 lower than those detected by the CTLL assay. A comparative study using a standard source of IL-2 demon-

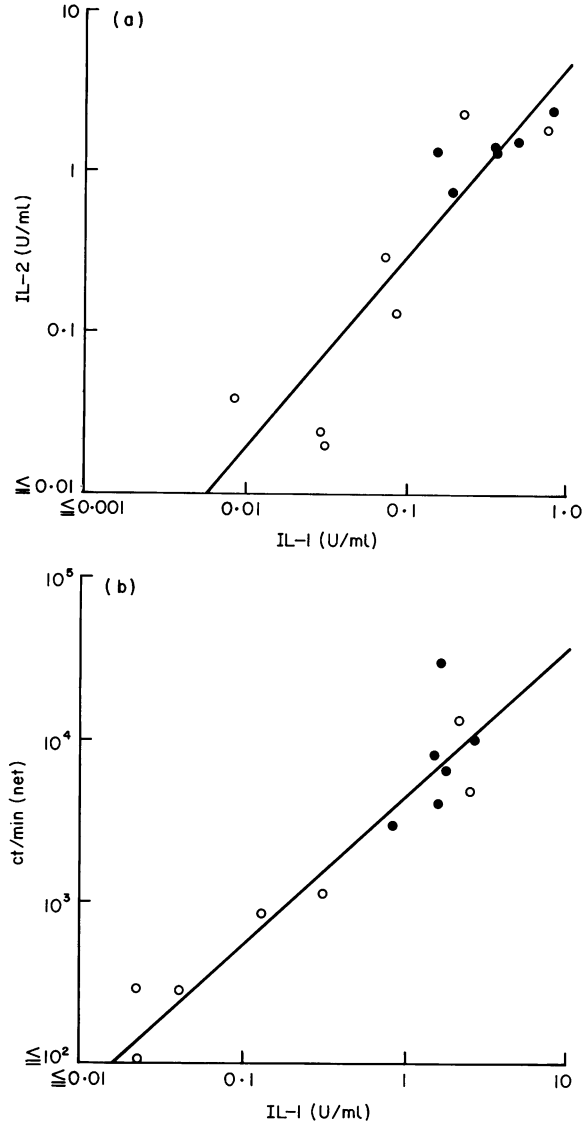

Fig. 1. (a) Correlation between interleukin-1 (IL-1) level measured at 12 h of culture and IL-2 concentration at 48 h in response to 0·05 μg/ml of phytohaemagglutinin (PHA). Open circles represent the response of subjects with HLA-B8/DR3 and closed circles represent individuals without these HLA antigens. (b) Correlation between IL-2 concentration measured at 48 h and the proliferative response determined at 72 h of culture. Open and closed circles as in (a).

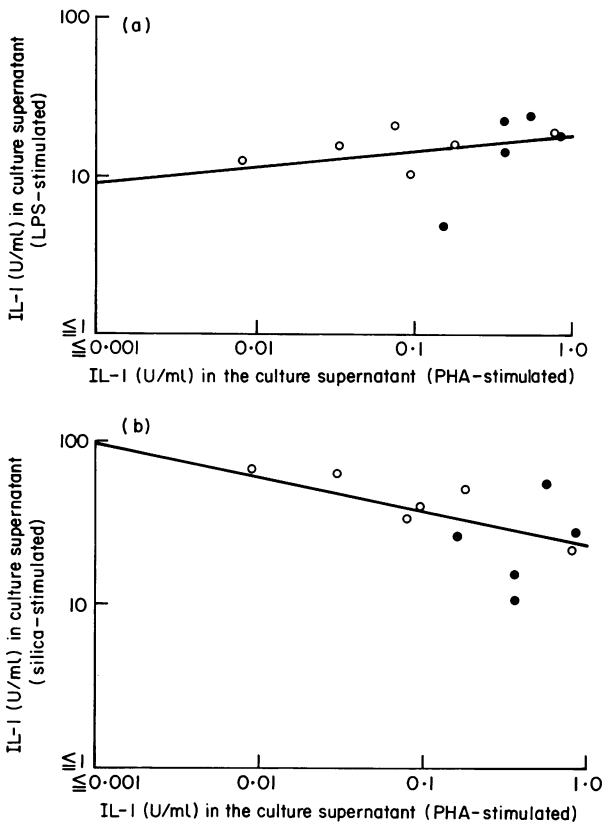

Fig. 2. Lack of significant correlations between IL-1 concentrations produced by peripheral blood mononuclear cells in response to 0·05 μg/ml of phytohaemagglutinin and monocytes purified from the same individual responding to (a) lipopolysaccharide; or (b) silica particles.

strated that our CTLL-2 assay has, in fact, more than an order of magnitude greater sensitivity to IL-2 than the mouse thymocyte assay (Table 2). Nonetheless, it was possible that very small levels of IL-2 (although undetectable by the CTLL assay) may act synergistically with IL-1 in culture supernatants and alter the results (Mannel *et al.*, 1985). This potential technical difficulty is also very improbable, as shown in Table 3, where the first column shows the response of thymocytes to several concentrations of IL-1 in the absence of added IL-2. It requires more than 0·25 U/ml of IL-2 to have even a minimally synergistic effect on the mouse thymocyte assay (Table 3), and concentrations of IL-2 in this range are well above the level of detection with our CTLL assay (Table 2).

The supernatant from a low responder subject with decreased IL-1 levels does not inhibit thymocyte proliferation induced by exogenous IL-1

We then examined the possibility that increased production of an IL-1 inhibitor may be responsible for the low measured levels of IL-1 activity in our subjects with HLA-B8/DR3. Several dilutions of supernatants from a very low responder subject were mixed with a standard IL-1 preparation to determine if there was inhibition of the thymocyte response to the exogenous IL-1 (Fig. 3). The response of the thymocytes to several concentrations of IL-1 (open circles) was not inhibited by any of several dilutions of the PHA supernatant of the low responder subject (closed circles, open and closed triangles).

IL-1 inhibitor levels of column-fractionated supernatants from low responder subjects with HLA-B8/DR3 have no significant increase in levels of IL-1 inhibitors compared with normal responder controls

Supernatants from mononuclear cell cultures of two low responder subjects with HLA-B8/DR3 and two high responder controls were collected after 12 h of stimulation with 0·05 μg/ml of PHA and fractionated on sephacryl-200 columns as previously described (Berman *et al.*, 1986). The fractions were analysed for IL-1 inhibitor activity by culturing with exogenous IL-1 in the mouse thymocyte assay. The inhibitor activity of the various fractions were quantified by plotting on paper and then cutting out and weighing the portion of the graph within the

Table 2. The CTLL-2 microassay is far more sensitive to IL-2 than the mouse thymocyte assay

Indicator cells	Medium alone (net ct/min ± s.e.m.)	Medium + IL-2 (U/ml)							
		0·003125	0·0625	0·125	0·25	0·5	1	2	4
CTLL-2	130 ± 5	324 ± 54	624 ± 89	2628 ± 146	6913 ± 127	13618 ± 382	20435 ± 968	21676 ± 296	25640 ± 296
Mouse thymocytes	80 ± 6	ND	ND	−4 ± 3	39 ± 13	368 ± 47	514 ± 90	1454 ± 190	9555 ± 422

ND, not determined.

Table 3. Synergistic effect of IL-1 and IL-2 on mouse thymocyte proliferation: effect of exogenous IL-2 on mouse thymocyte co-stimulation assay for IL-1

IL-1 (U/ml)	IL-2 (U/ml)					
	Nil	0·125	0·25	0·5	1	2
Nil	483 ± 24	0	−22 ± 15	65 ± 82	534 ± 141	1857 ± 30
0·125	2496 ± 106	2433 ± 104	2669 ± 200	4722 ± 414	7995 ± 283	15995 ± 154
0·25	7088 ± 501	6360 ± 440	6175 ± 126	7610 ± 353	14851 ± 765	23957 ± 154
0·5	11509 ± 1267	11014 ± 610	11438 ± 1146	13947 ± 1029	18998 ± 231	29625 ± 1235
1	14432 ± 690	13204 ± 1328	14849 ± 1012	16774 ± 401	20957 ± 445	34844 ± 48

Results are net ct/min after subtraction of 483 ct/min ± s.e.m.

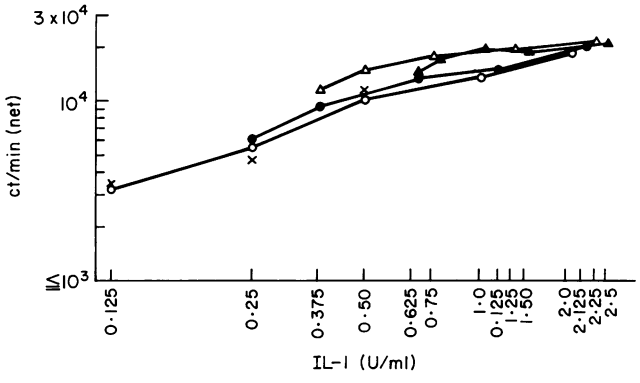

Fig. 3. Lack of an inhibitory effect of a PHA-stimulated supernatant from a low responder subject with HLA-B8/DR3 on the mouse thymocyte response to several concentrations of IL-1. A supernant dilution (×) of 1:8 stimulated the same response as 0·125 μg/ml of exogenous IL-1, 1:4 the same as 0·25 μg/ml and 1:2 the same as 0·5 μg/ml. The thymocyte response to IL-1 + supernatant was the same as would be predicted for an additive effect of IL-1 from both sources. ○, IL-1 alone; ●, IL-1 + 1:8 diluted supernatant; △, IL-1 + 1:4 dilution; and ▲, IL-1 + 1:2 dilution.

inhibitor range. The relative inhibitory activity of the high responder subjects had a mean of 12·7 ($g \times 10^{-3}$) compared with only 8·0 for the low responders.

DISCUSSION

IL-1 is a 17000 *Mr* peptide that is produced in a variety of tissues and has many biological effects (Reviewed by Dinarello & Mier, 1987), including fever production, augmentation of hepatic protein synthesis and vascular permeability effects. From an immunological standpoint, one of the best known functions of IL-1 is its action as a co-stimulant for the production of IL-2 by T lymphocytes. IL-1 is produced very early in an immune response by accessory cells, for example, of the monocyte/macrophage series and acts as a major regulatory factor on T cell proliferation and IL-2 production.

In the studies reported here, we examined the role of IL-1 in the impaired lymphocyte response to mitogens that is associated with the histocompatibility antigens HLA-B8 and DR3. We have previously demonstrated that impaired production of IL-2 is a major factor in the poor responsiveness of lymphocytes from individuals with HLA-B8/DR3 to suboptimal concentrations of PHA (Hashimito *et al.*, 1989). Our present data demonstrate that impaired IL-2 production is associated with, and may be due in part to impaired IL-1 production. As a group, low responder subjects with HLA-B8/DR3 have an impaired proliferative response to suboptimal concentrations of PHA at 72 h of culture that is comparable to their decreased IL-2 accumulation at 48 h. That, in turn, is comparable to their decreased IL-1 accumulation at 12 h. In addition, there is a strong correlation between a specific individual's IL-1 concentration at 12 h of culture and that individual's subsequent IL-2 concentration at 48 h. There is a similar association between the IL-2 concentration and the proliferative response at 72 h. These results are consistent with a model involving a defect in activation events occurring prior to the production of IL-1 that leads to decreased IL-1 production, and therefore impaired IL-2 production and lymphocyte proliferation. An alternative explanation, that our subjects actually have an increased production of an IL-1 inhibitor, is very unlikely as we were not able to demonstrate increased inhibitory activity in either untreated or column-fractionated supernatants from low responder subjects.

Previous studies have shown impaired release of IL-1 in patients with systemic lupus erythematosus (Linker-Israeli *et al.*, 1983) and scleroderma (Sandborg *et al.*, 1985), and increased release of this monokine in *Yersinia* arthritis patients and HLA-B27 positive control subjects (Repo *et al.*, 1988). These abnormalities involved the release of IL-1 from monocytes directly stimulated with LPS or phorbol esters and thus differ significantly in mechanism from the impaired IL-1 production of our subjects. The low responder subjects with HLA-B8/DR3 had decreased IL-1 production only in a culture system using suboptimal concentrations of PHA, a T cell mitogen. Although the precise stimulus for IL-1 production in this situation is unclear, it is likely to be dependent on a T cell signal (Mizel, 1982; Weaver & Unanue, 1986) that involves direct cell–cell contact or release of another cytokine such as tumour necrosis factor (Dinarello *et al.*, 1986) or interferon (Arenzana-Seisdedos, Virelizier & Fiers, 1985) even earlier in the immune response. Since the proliferative response to suboptimal concentrations of mitogen is especially dependent on the interaction of T cells and accessory cells (McCombs, Michalski & Talal, 1976; Roosnek, Brouwer & Aarden, 1985), our results are consistent with an abnormality of the initial interaction of T cells and accessory cells or some other very early event that is required for a cellular immune response.

The precise clinical significance of the abnormality of T cell activation associated with HLA-B8/DR3 is unclear. Initially, we found an HLA-associated impaired proliferative response in Sjögren's syndrome patients with HLA-B8 (Michalski *et al.*, 1978), and subsequently a similar abnormality was reported in scleroderma patients with HLA-B8/DR3 (Kallenberg *et al.*, 1981). Since essentially the same abnormality also occurs in normal individuals with HLA-B8/DR3, but without evidence of autoimmunity (McCombs & Michalski, 1982), it presumably preceeds the autoimmune disease, and may reflect an alteration of immune regulation that predisposes to immune-mediated disease.

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