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Defective antigen processing associated with familial disseminated mycobacteriosis

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SUMMARY

To gain insights into a possible immune defect predisposing to disseminated mycobacteria infection, we studied three of six surviving children with disseminated *Mycobacterium avium* complex infection, who had no recognized form of immunodeficiency. We used mycobacteria isolated from the patients and diphtheria, tetanus and pertussis vaccine (DTP) to study antigen-specific T lymphocyte responses. We observed that interferon-gamma (IFN-γ) production by T cells in response to antigens (both mycobacteria and DTP) in these patients with disseminated infection was greatly impaired. This defect did not seem to be the result of T cell unresponsiveness, as phytohaemagglutinin (PHA) stimulation was able to induce high levels of IFN-γ comparable to those seen in control patients with localized infection. Further experiments showed that peripheral blood mononuclear cells (PBMC) from patients with disseminated infection were able to present influenza haemagglutinin (HA) peptides to specific T cell clones. However, this ability was lost when the whole HA protein was used as source of antigen. Taken together, these observations support the notion that the primary immune defect in these patients with disseminated mycobacterial infection rests in the antigen-processing functions of their antigen-presenting cells (APC). These findings may provide clues to the wider problem of susceptibility to mycobacteria and other intracellular pathogens and have implications in designing therapy for these patients.

Keywords *Mycobacterium avium* complex mycobacteria T cells interferon-gamma antigen processing

INTRODUCTION

We have recently reported a rare immunodeficiency in six children predisposing to disseminated *Mycobacterium avium* complex (MAC) infections [1]. The patients are from related families that live in Malta. The parents of patients and other family members had no history of mycobacterial infection, but low lymphoproliferation to mycobacteria was observed [1]. The children presented with fever, weight loss, anaemia, lymphadenopathy and hepatosplenomegaly. The infections were refractory to conventional therapy, although treatment with interferon-gamma (IFN-γ) was associated with improvement.

Organisms of the MAC are facultative intracellular parasites found within macrophages. Treatment of infections caused by MAC bacteria is difficult, as they are often resistant to antimycobacterial drugs [2,3]. *In vitro* studies have demonstrated that the ability of human macrophages to kill intracellular pathogens such as *Myco. -avium, -intracellulare* (MAI) is enhanced by IFN-γ [4,5]. In leprosy and tuberculosis also, IFN-γ is associated with increased mycobactericidal potential of the monocyte, and this effect was shown to be counterbalanced by IL-4 and IL-10 [6,7].

Disseminated MAC infections are rare in normal individuals but occur frequently in individuals with HIV infection [8]. However, disseminated mycobacteriosis has been reported previously in adults with no recognized form of immunodeficiency [9–11]. To gain insights into the primary immune defect predisposing to disseminated mycobacteriosis, we analysed patients with disseminated infection, children with localized infection and healthy individuals for the capacity of their T lymphocytes to proliferate and produce cytokines in response to antigenic stimuli presented via antigen-presenting cells (APC) or to mitogen (phytohaemagglutinin (PHA)) known to stimulate lymphocytes independently of antigen presentation. The antigen-processing function of APC from patients was assessed by analysing the capacity of isolated peripheral blood mononuclear cells (PBMC) to present influenza haemagglutinin antigen to specific T cell clones.

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PATIENTS AND METHODS

Patients

The three patients used in this study were children from related families in Malta who developed disseminated MAI complex infections (D-patients) in early childhood. These patients had not been immunized with bacille Calmette–Guérin (BCG) but had received diphtheria, tetanus, and pertussis (DTP) vaccination. Histology of biopsy material showed poor granuloma formation, and, besides mycobacteria, no other pathogen was cultured from their peripheral blood, spleens or lymph nodes.

The controls (L-patients) were children from the UK who had not been BCG vaccinated and had localized mycobacterial infection or other localized infections. Ethical committee approval was obtained before starting this study.

Antigens

Mycobacteria. Mycobacterial isolates were cultured from the blood of patients in Middlebrook's 7H9 broth (Difco Labs, Detroit, MI). Bacteria were harvested 7 days following culture with *Myco. fortuitum* and 15–21 days following culture in the case of *Myco. avium* or MAI, respectively, by centrifugation at 4000 rev/min for 30 min. The mycobacteria were resuspended in growth medium and cell count determined by the growth of colonies on Middlebrook's 7H11 agar plates. Heat-killed preparations of mycobacteria were obtained by heating 10^7 organisms/ml PBS in a water bath at 70°C for 70 min. They were stored at –20°C.

DTP vaccine. DTP vaccine obtained from Wellcome Laboratories (Beckenham, UK) was used at two dilutions, 1 : 4000 and 1 : 8000, of the stock (0·5 ml containing 30 U of diphtheria, 60 U of tetanus and 4 U of *Bordetella pertussis*). It was used as a third party antigen.

Influenza virus haemagglutinin. Influenza virus haemagglutinin (HA) purified from recombinant X-31 virus was a generous gift from Dr J. Skehel (National Institute for Medical Research, Mill Hill, UK). The HA peptides (residues 307–319 and residues 255–270) were synthesized using standard solid-phase methods as described previously [12].

Cytokine assay

Supernatants from 10^6 PBMC stimulated either with heat-killed mycobacteria (10^6/ml) or PHA (1 μg/ml) for 3 days, were tested. Cytokines released in culture supernatants were assayed by cytokine-specific ELISA as described previously [13]. The sensitivity of the assays was as follows: IFN-γ, 100 pg/ml [14]; IL-2, 10 pg/ml [15]; IL-4, 50 pg/ml [16]; IL-5, 10 pg/ml [17]; IL-10, 40 pg/ml [13].

Assay for T cell proliferation and IFN-γ production

PBMC (10^6/ml) from patients and controls were stimulated with heat-killed mycobacteria (10^6/ml) or DTP (1 : 400/800) in a standard proliferation assay. Three days later the microtitre plate was centrifuged (90 ***g***) and 100 μl of supernatant were harvested from each well and transferred into an identical microtitre plate. The microtitre plate with cells was then replenished with 100 μl/well of fresh medium and cells kept in culture till day 6 were pulsed with ^{3}H-thymidine (^{3}H-TdR) for 18–20 h. Plates were harvested the next day and proliferation was determined. Supernatants from microtitre well cultures stimulated with mycobacteria/DTP giving highest proliferation, were analysed for IFN-γ production. In each case T cell proliferation and IFN-γ production on PHA stimulation was used as a positive control. IFN-γ was assayed using an ELISA Kit from Endogen Inc (Boston, MA) that had a sensitivity of 5 pg/ml.

Assay for antigen processing and presentation by cells from D-patients

PBMC of the three D-patients used in these experiments were typed for their MHC class II haplotype. D-patient 1 was DR1,7 DR53 DQ2,5; D-patient 2 was DR11,6 DR52 DQ1,3; and D-patient 3 was DR11,14 DR52 DQ5,7. PBMC and Epstein–Barr virus (EBV)-transformed B cells from patients were used as APC after irradiation at 30 Gy and 60 Gy, respectively. The T cell clone HA1.7 [18] specific for influenza HA peptide (residues 307–319) is restricted by DRb1*0101 (DR1), therefore APC from D-patient 1 were used to present HA-derived peptide to this clone. T cell clone PF5 is specific to influenza HA peptide (residues 255–270) (A. Faith and J.R. Lamb, unpublished results) and is restricted by DRb1*1101(DR11), therefore APC from D-patients 2 and 3 were used to present HA-derived peptide to PF5. The APC and T cell clone were used at a 1:1 ratio of 2×10^4 T cells and 2×10^4 APC/well of a microtitre plate. PBMC from healthy individuals expressing the MHC class II restricting allele matched with the T cell clones (a gift from Dr René de Vries, University Hospital, Leiden, The Netherlands) were used as a positive control. For experiments done using EBV-transformed B cells from D-patients as APC, the positive controls used were L-NAT (an EBV-transformed B cell line derived from the donor of the clone HA1.7) for D-patient 1 and SWEIG (an EBV-transformed typing B cell line which is homozygous for DRb1*1101) for D-patients 2 and 3. Cells were labelled with ^{3}H-TdR after 48 h in culture and harvested 18–20 h later.

RESULTS

Cytokine production in response to mycobacterial antigens and PHA

Supernatants from cultures of PBMC from D- and L-patients stimulated with PHA or heat-killed mycobacteria were analysed for the presence of different cytokines (Fig. 1). PHA was used in order to test the capacity of T cells to produce cytokines in response to a stimulus independent of antigen presentation. As seen in Fig. 1a, PHA was able to stimulate T cells from D-patients to produce IFN-γ as well as IL-2, IL-4, IL-5 and IL-10 at high levels comparable to those released by T cells from L-patients. In contrast, stimulation with mycobacteria was not able to induce IFN-γ production by T cells from D-patients 1 and 2, although it stimulated IFN-γ from T cells of L-patients (Fig. 1b). Interestingly, IL-4 and IL-10 also were not produced in these patients. D-patient 3, on the other hand, who was showing good clinical recovery, produced amounts of IFN-γ, IL-4 and IL-10 comparable to those produced in L-patients. Finally, low amounts of IL-2 and IL-5 comparable to L-patients were produced by T cells from all D-patients (Fig. 1b).

T cell proliferation and IFN-γ production to mycobacterial antigens and third party antigen

The purpose of these experiments was to examine whether T cells from D-patients that did not produce IFN-γ in response to mycobacteria could proliferate and secrete IFN-γ following stimulation with DTP. As expected in response to mycobacteria-derived antigens, T cell proliferation and IFN-γ production was poor in D-patients (except in D-patient 3) compared with controls (Fig. 2). The third-party antigen, DTP, also induced poor

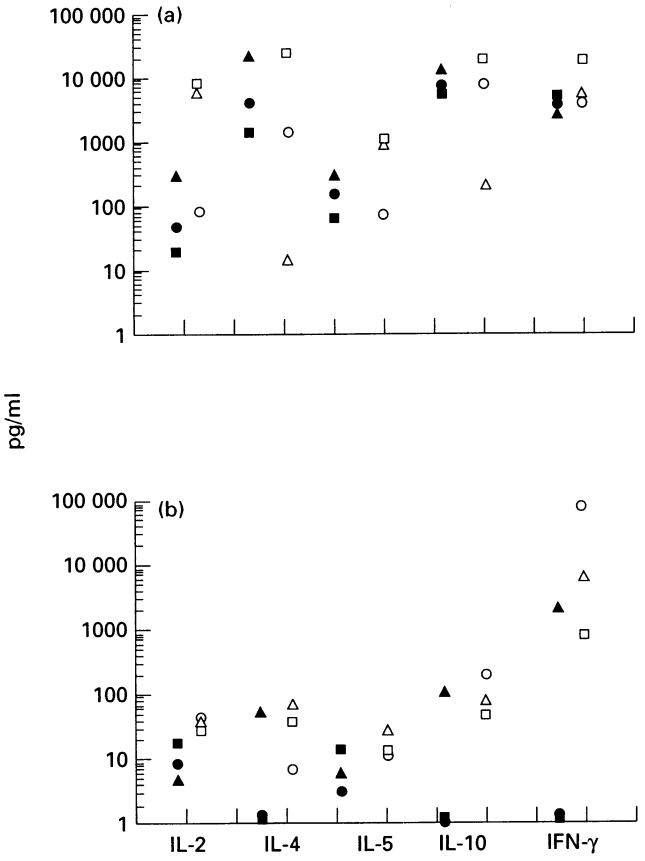

Fig. 1. Cytokine production in patients with disseminated (D-patients) and localized (L-patients) mycobacterial infection. (a) Cytokines produced 3 days after stimulation with phytohaemagglutinin (PHA; 1 μg/ml). (b) Cytokines produced 3 days after stimulation with mycobacteria. Peripheral blood mononuclear cells (PBMC; 10^6/ml) from each D-patient were stimulated with heat-killed mycobacteria (10^6/ml) isolated from the blood of the respective patient. PBMC from L-patients were stimulated with heat-killed *Mycobacterium avium* (strain 10763). Cytokines secreted in 3-day culture supernatants were measured by cytokine-specific ELISA. ●, D-patient 1; ■, D-patient 2; ▲, D-patient 3; ○, L-patient 1; □, L-patient 2; △, L-patient 3.

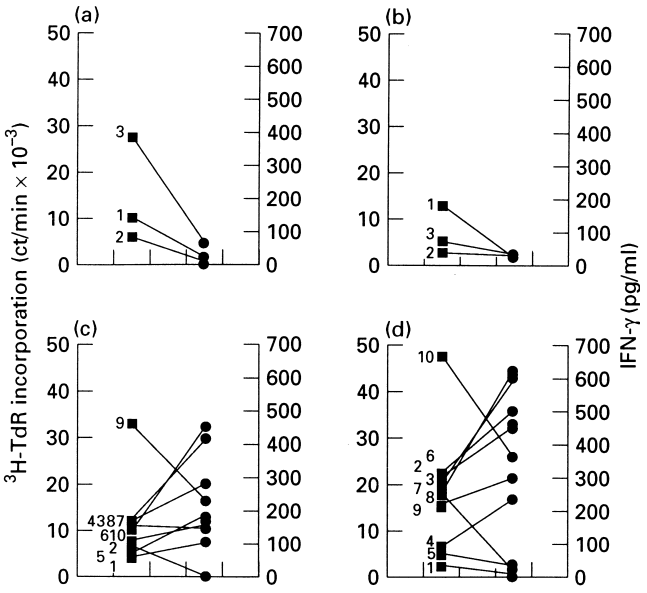

Fig. 2. Lymphoproliferation and IFN-γ production following stimulation with mycobacteria (a,c) and diphtheria, tetanus and pertussis (DTP) (b,d) in patients with disseminated mycobacterial infection (D-patients) and controls. Culture supernatant (100 μl) corresponding to the dose of mycobacterial antigen or DTP giving optimum T cell proliferation was analysed. The amount of IFN-γ produced was assayed using a cytokine-specific ELISA. Controls (c,d) used were age-matched children with localized mycobacterial or non-mycobacterial infections. Peripheral blood mononuclear cells (PBMC) from controls were treated identically to patients' cells. Numbers in figure refer to number of D-patient/number of control. Phytohaemagglutinin (PHA)-induced proliferation of cells from patients and controls was in the range of 35 453–65 786 ct/min, and IFN-γ produced was 550–900 pg/ml. Details of protocol in Patients and Methods.

proliferation and poor IFN-γ production (< 50 pg/ml) in T cells from all D-patients. In contrast, seven out of the 10 controls (Fig. 2b,d) were able to produce high amounts of IFN-γ (> 230 pg/ml). Cells stimulated with PHA (used as positive controls for the experiment) from both D-patients and controls proliferated strongly and produced high levels of IFN-γ (data in legend of Fig. 2).

Processing of influenza HA protein by APC from D-patients

In order to investigate whether the impaired IFN-γ production and poor proliferative response observed in D-patients following antigenic stimulation was the result of a defective antigen-processing function, we examined the ability of PBMC and EBV-transformed B cells isolated from D-patients to process and present HA protein to specific $CD4^+$ T cell clones. HLA-DR1-restricted T cell clone HA1.7, specific to HA peptide residues 307–319, was used to test APC from D-patient 1, and HLA-DR11-restricted T cell clone PF-5, specific to HA peptide residues 255–270, was used to test APC from D-patients 2 and 3. Control APC were PBMC and EBV-transformed cell lines derived from HLA-DR-matched healthy non-infected individuals (see Patients and Methods).

As seen in Table 1, transformed B cell lines from D-patients 1 and 2 were capable of inducing proliferation of HA-specific T cell clones as efficiently as control transformed B cells. EBV lines from D-patient 3, although stimulating weaker proliferative responses than control lines, demonstrated the ability to process and present the HA protein. For all the EBV lines however, the two synthetic peptides of HA protein usually induced better proliferative responses than the whole HA molecule. PBMC from D-patients also showed a good capacity to present antigen to specific T cell clones, as seen by the level of ^{3}H-TdR incorporation induced when HA peptides were used as source of antigen (Table 1). However, in assays where the whole HA protein was used as antigen, the capability of D-patients' PBMC to stimulate proliferation of HA-specific clones was completely absent with ^{3}H-TdR incorporation dramatically reduced to background levels (Table 1). Thus, although PBMC from D-patients are able to efficiently present synthetic peptides spanning the epitopes recognized by the HA-specific T cell clones, they appear to lack the antigen-processing function.

DISCUSSION

Understanding the cellular mechanisms that predispose to infections is of prime importance to gain insights into susceptibility to

Table 1. Processing and presentation of influenza haemagglutinin (HA) protein by peripheral blood mononuclear cells (PBMC) and Epstein–Barr virus (EBV)-transformed B cells from patients who had received diphtheria, tetanus and pertussis vaccination (D-patients)

		Proliferative response of influenza HA-specific T cell clones* (ct/min $\times 10^{-3}$) to:		
Patients	Antigen-presenting cells	Medium	HA Peptide (1 μg/ml)	Whole HA protein (10 μg/ml)
D-patient 1	Transformed B cells	1·3	95·4	24·8
Control†	Transformed B cells	2·2	104·1	30·7
D-patient 1	PBMC	0·3	25·9	ND
Control‡	PBMC	0·7	25·8	ND
D-patient 2	Transformed B cells	1·1	94·5	36·2
D-patient 3	Transformed B cells	3·5	22·8	5·2
Control§	Transformed B cells	4·5	86·6	88·8
D-patient 2	PBMC	0·3	13·3	0·1
D-patient 3	PBMC	1·3	18·6	0·2
Control¶	PBMC	0·2	27·7	13·3

* T cell clone HA 1.7 specific for influenza HA recognizes peptide residues 307–319 and shares the DR haplotype with D-patient 1. T cell clone PF-5 specific for influenza HA recognizes peptide residues 255–270 and shares its DR haplotype with D-patients 2 and 3.

† LNAT, an EBV cell line.

‡ PBMC from normal donor with same DR allele as D-patient 1.

§ SWEIG, an EBV cell line.

¶ PBMC from normal donor with same DR allele as D-patients 2 and 3.

disease. Towards this aim we studied immune responses in children with disseminated MAC infections. In an attempt to identify the primary cellular defect in these patients, T lymphocytes were tested for their ability to proliferate and to secrete cytokines in response to stimulation by protein antigens as well as by mitogen. Impaired T cell proliferation and secretion of cytokines, especially IFN-γ, was observed in response to both mycobacteria-derived antigen and third-party antigen.

Mycobacterial infections have been associated with impaired IFN-γ production. In murine studies, animals with a targeted disruption of the IFN-γ gene were shown to be killed by sublethal doses of *Myco. bovis* [19]. Low IFN-γ levels in T cell cultures stimulated with autologous *Myco. tuberculosis*-infected monocytes have also been reported in TB patients [20]. In lepromatous leprosy and in allergic conditions diminished IFN-γ production by T cells following antigenic stimulation has also been reported [6,7]. In these latter studies, impaired IFN-γ production was associated with high secretion of IL-4 and IL-10 resulting from skewing of the cytokine profile towards a Th2 functional phenotype. This does not seem to be the case in our patients, as both IL-4 and IL-10 were also not secreted by T cells after exposure to mycobacteria and DTP. Holland *et al.* reported a reduced capacity to secrete IFN-γ by mitogen-stimulated T cells in adult patients with familial MAC infection [9]. This opposes our results, where T cells from patients with disseminated mycobacteria infection responded normally to PHA by secreting high amounts of IFN-γ as well as other cytokines. This discrepancy is suggestive of a difference in the mechanisms responsible for the immunodeficiencies characterizing the two groups of patients. In the patients studied by Holland *et al.* the lack of responsiveness to mitogen might imply a primary defect in the responding T cell population. In contrast, in our patients, T cell responsiveness to PHA excludes the possibility of a defect at the T cell level and suggests a possible deficiency in the mechanisms responsible for antigen processing and/or antigen presentation to T cells.

The hypothesis of defective antigen processing rather than defective antigen presentation responsible for the lack of T cell responsiveness to antigen was confirmed by the observation that PBMC from our patients, while being able to stimulate antigen-specific T cell clones when cultured with antigen-derived peptides, were inefficient stimulators in the presence of the whole protein antigen. This antigen-processing defect did not seem to be due to possible mutations in genes involved in antigen processing, since EBV-transformed B cells derived from the same patients were capable of processing native protein antigens. Alternatively, defective expression of these genes in professional APC is probable. The presence of an antigen-processing deficiency in monocytes is relevant to the disease, as they are the predominant hosts to mycobacteria *in vivo*. This, however, needs to be confirmed. At this stage the precise pathway within the complex antigen-processing machinery that is defective in patients also remains to be elucidated.

Finally, to our knowledge there is no previous report on defective antigen processing in disseminated mycobacteriosis. One could envisage a genetic basis to this familial defect, though its analysis is beyond the scope of this study. However, the antigen-processing defect in patients is very reminiscent of the defect expressed in the monocytes of mice, induced by the murine *Bcg* susceptibility gene [21–23]. Although a human candidate of the *Bcg* susceptibility gene has been recently mapped [24], its function is unknown. However, linkage between the human locus and the defect in these children was not demonstrable (Newport *el al.*,

unpublished observations). Nevertheless, mutations in alternative genes, by analogy to cellular defects in activation and signal transduction in chronic granulomatous disease [25] and defective killing of intracellular parasites by mice lacking the 55-kD tumour necrosis factor (TNF) receptor [26], are being investigated.

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