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Predominant Recognition of the ESAT-6 Protein in the First Phase of Infection with *Mycobacterium bovis* in Cattle

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Tuberculosis continues to be a worldwide health problem for both humans and animals. The development of improved vaccines and diagnostic tests requires detailed understanding of the immune responses generated and the antigens recognized during the disease. This study examined the T-cell response which develops in cattle experimentally infected with *Mycobacterium bovis*. The first significant T-cell response was found 3 weeks after the onset of infection and was characterized by a pronounced gamma interferon (IFN-γ) response from peripheral blood mononuclear cells directed to antigens in culture filtrates. Short-term culture filtrate (ST-CF) was separated into molecular mass fractions and screened for recognition by T cells from experimentally infected and field cases of bovine tuberculosis. Cattle in the early stages of experimental infection were characterized by strong IFN-γ responses directed predominantly toward the lowest-mass (<10-kDa) fraction of ST-CF, but cattle in later stages of experimental infection (16 weeks postinfection) exhibited a broader recognition of antigens of various molecular masses. Field cases of bovine tuberculosis, in comparison, preferentially recognized low-mass antigens, characteristic of animals in the early stages of infection. The major T-cell target for this dominant IFN-γ response was found to be the secreted antigen ESAT-6. This antigen was recognized strongly by the majority of field cases of bovine tuberculosis tested. As ESAT-6 is unique to pathogenic mycobacterial species, our study suggests that ESAT-6 is an antigen with major potential for vaccination against and specific diagnosis of bovine tuberculosis.

Human tuberculosis and animal tuberculosis continue to be major health problems on a worldwide scale. The human disease is responsible for approximately 3 million deaths annually (38), while tuberculosis in cattle is a major cause of economic loss and represents a significant cause of zoonotic infection (14). Both diseases are caused by closely related mycobacterial species within the tuberculosis complex, with *Mycobacterium tuberculosis* and *M. bovis* the main causative agents in humans and cattle, respectively. Only very limited differences in the antigens expressed by strains within the tuberculosis complex have been found, whereas the expression of a number of antigens clearly dissociate these strains from nonpathogenic strains (2, 3, 18). In recent years, there has been a considerable research effort for these diseases, but major gaps in our understanding of the basic parameters in the host-pathogen relationship still exist. It is evident that such information is needed in rational attempts to develop improved vaccines and diagnostic reagents.

It is an established fact that cell-mediated responses are responsible for immunity to tuberculosis (24, 26). Several studies of animal models of tuberculosis have demonstrated the importance of $CD4^+$ cells (22, 29) and their production of gamma interferon (IFN-γ) (13, 16) for protection against tuberculosis. Recently, knockout-mouse models have allowed the sensitive monitoring of the influences of other T-cell populations, and it appears that both $CD8^+$ and to a lesser extent γδ T cells have roles in the immune response to mycobacteria (17, 21). Recent work with cattle has confirmed that all of these cells are involved in natural tuberculosis infection and has suggested a dynamic change in the dominant subpopulation as the disease progresses (33).

While detailed knowledge of the T-cell subpopulations involved in immunity to tuberculosis is increasing, information on the mycobacterial antigens recognized during the course of infection is still limited. Antigen recognition at various stages of infection has been the subject of a number of studies with the mouse model of tuberculosis infection. In these studies, culture filtrate antigens were found to contain major target molecules recognized by T cells at various stages of infection (6, 7). The division of culture filtrate into narrow fractions allowed the identification of the secreted antigens ESAT-6 and Ag 85 as major target molecules recognized by T cells involved in protective immunity (9). The relevance of these findings for antigen recognition in different phases of tuberculosis infection in humans has obviously been very difficult to investigate in a controlled manner. In the present study, the development of T-cell responses in a natural host has been addressed with an experimental model of tuberculosis established in cattle. An investigation of the T-cell responses and mycobacterial antigens recognized throughout infection revealed a pattern which changed as the disease progressed. The low-mass secreted antigen ESAT-6 was found to be a dominant IFN-γ target in the early phase of infection, whereas several antigens of different molecular masses were recognized to a similar degree later in experimental infection. Importantly, field cases of tuberculosis shared the preferential recognition of low-mass antigens characteristic of animals in the early stages of infection. The broad recognition of ESAT-6 in these animals points to its potential importance as a candidate antigen for vaccines and diagnostic reagents.

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MATERIALS AND METHODS

Skin test responses. Cattle were skin tested with a single intradermal comparative cervical test where the responses to purified protein derivatives (PPD)

prepared from *M. bovis* (PPDB) are compared with the responses to PPD prepared from *M. avium* (PPDA) (14a). Volumes (0.1 ml) of PPDB (1.0 mg/ml) and PPDA (0.5 mg/ml) were injected intradermally at separate sites in the cervical region after the skin thickness (in millimeters) had been measured with callipers. The increases in skin thickness were subsequently measured at 72 h after intradermal injections.

Animals. Cattle of two types were used in these experiments.

(i) Experimentally infected cattle. Groups of Friesian-cross animals of approximately 9 months of age were obtained from a herd with no history of *M. bovis* infection for at least 5 years. These animals were skin test negative at the outset of the experiment. The animals were placed in strict isolation and infected by intranasal instillation of approximately 5×10^6 CFU of a strain of *M. bovis* (T/91/1378; Veterinary Sciences Division, Belfast, United Kingdom) which had been isolated from a field case of bovine tuberculosis and minimally passaged. The methods of infection and housing have been described in detail previously (27).

(ii) Field skin test-positive cattle. Blood samples were collected from skin test-reactive animals which had responses biased toward PPDB compared with PPDA. Nineteen samples were obtained from a herd of 113 animals with a 28% skin test-reactive rate. Blood samples were collected within 1 week of the positive skin test, and 11 of the 19 animals sampled had gross tuberculosis lesions when they were examined postmortem at the abattoir. *M. bovis* infection was confirmed by histopathology.

Mycobacterial antigens. (i) ST-CF. Short-term culture filtrate (ST-CF) was produced as described previously (8). Briefly, *M. tuberculosis* (8×10^6 CFU) was grown on modified Sauton medium without Tween 80 on an orbital shaker for 7 days. Culture supernatants were sterile filtered and concentrated, and the buffer was exchanged with phosphate-buffered saline (PBS) on a YM3 membrane (Amicon, Denver, Mass.).

(ii) Molecular mass fractions of ST-CF. Culture filtrate proteins were divided into narrow molecular mass fractions by elution of polyacrylamide gels as described previously (5). In brief, ST-CF (5 mg of protein) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 to 20% polyacrylamide gel) overnight. The gel was transferred to a whole-gel eluter unit (Bio-Rad, Richmond, Calif.) and electroeluted (40 V) for 20 min. Protein fractions were harvested, and the purity and molecular mass range covered by each fraction was analyzed by SDS-PAGE (10 to 20% polyacrylamide), followed by silver staining (10). The protein concentrations in different fractions were estimated by the micro bicinchoninic acid method (Pierce, Oud-Beijerland, The Netherlands). The 30 fractions were pooled to give a total number of 17, and they were all adjusted to isotonia with concentrated PBS and stabilized with 0.5% fetal calf serum. All fractions were kept frozen at −80°C until used. None of the fractions were toxic in cell cultures.

(iii) ESAT-6. Native ESAT-6 was purified from ST-CF by conventional biochemical techniques described in detail elsewhere (35). Recombinant ESAT-6 (rESAT-6) was produced with the pMAL-p expression vector (New England Biolabs, Beverly, Mass.) as previously described (28). Purification of the rESAT-6 fusion protein could not be accomplished by conventional affinity chromatography on an amylose resin and was therefore done by preparative SDS-PAGE (12% acrylamide) with a Prep-Cell apparatus (Bio-Rad) according to the manufacturer's instructions. SDS was removed by passage through detergent binding columns (Extractigel; Pierce), and the buffer was exchanged with PBS before its use in cell cultures.

(iv). PPD. PPDB and PPDA were obtained from Central Veterinary Laboratories, Weybridge, United Kingdom.

IFN-γ blood test. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples by density centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden), washed by centrifugation in PBS, and resuspended in complete medium at a concentration of 10^6 cells/ml. Complete medium comprised RPMI 1640 containing HEPES buffer, 2 mM L-glutamine, 25 μg of gentamicin sulfate per ml, and 5% fetal calf serum (Gibco, Paisley, United Kingdom). Cultures of PBMC were established in microtiter plates with flat-bottomed wells and stimulated with antigen or concanavalin A (Sigma Chemical Company, Poole, United Kingdom) as a positive control for cell viability. All antigens, including ST-CF fractions, were used at a standard concentration of 2 μg/ml. IFN-γ responses were measured as described previously (32). In brief, after 24-h incubation at 37°C in 6% CO_2, supernatants were harvested from stimulated and control cultures and transferred to wells of a two-antibody enzyme-linked immunosorbent assay (ELISA) specific for bovine IFN-γ (Commonwealth Serum Laboratories, Parkville, Australia). The results were expressed as optical density indices (ODI) as follows: ODI = OD for stimulated wells/OD for control wells. For the purposes of this study, an ODI of ≥2 was used as an arbitrary indication of a positive response. The coefficient of variation between duplicate wells was <5%, and the OD for control wells was usually <0.1.

Statistics. The correlation between responses to two antigens was determined by linear regression by the sum-of-least-squares method. Mean responses to individual antigens were compared by the paired Student *t* test.

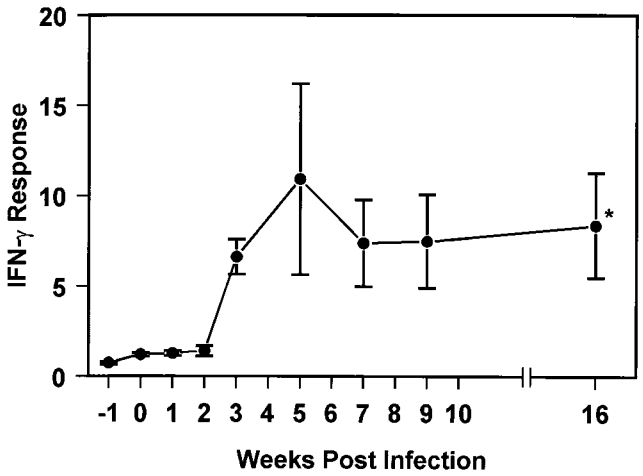

FIG. 1. Development of T-cell responses to mycobacterial antigens during experimental infection with *M. bovis*. Skin test-negative cattle from a tuberculosis-free herd were infected with virulent *M. bovis* by intranasal instillation of approximately 5×10^6 CFU, and the induction of T-cell responses to PPDB was monitored over the course of infection. PBMC were isolated at various time points, and responses were measured as in vitro release of IFN-γ. Data are means ± standard errors of the means ($n = 4$) of ODI. *, data at 16 weeks p.i. obtained from separate animals ($n = 3$) in a parallel experiment.

RESULTS

Development of T-cell responses during infection with *M. bovis*. A group of skin test-negative animals obtained from a herd with no history of bovine tuberculosis was used for the study of experimental tuberculosis infection. The in vitro reactivity of these animals to mycobacterial antigens was tested before the start of the experiment, and no recognition of PPDB was found prior to experimental infection (Fig. 1). Animals were infected by intranasal instillation of virulent *M. bovis* and monitored over a period of 16 weeks for in vitro T-cell responses to PPDB (Fig. 1). These animals were confirmed to have been infected by a combination of gross pathology, histopathology, and culture of *M. bovis* from tissues after the termination of the experiment. Antigen-specific IFN-γ release was used as a measure of T-cell reactivity and could be detected from 3 weeks postinfection (p.i.). Peak responses were found at week 5 p.i., followed by a gradual decline in activity to a somewhat lower level which remained throughout the experiment.

The antigens recognized by T cells change throughout the course of tuberculosis infection. Culture filtrate antigens have previously been demonstrated to be recognized at early time points in studies of experimental tuberculosis in animal models (7, 9). ST-CF is a complex mixture of culture filtrate proteins which by SDS-PAGE separation is seen to comprise 30 to 40 major bands, including a number of previously characterized mycobacterial proteins shared by closely related strains within the tuberculosis complex (Fig. 2). The antigens recognized by animals infected with tuberculosis were mapped by stimulating PBMC with a panel of narrow molecular mass fractions prepared from ST-CF by elution of proteins separated on polyacrylamide gels. This procedure enables the separation of complex protein mixtures into narrow molecular mass fractions suited for specificity analyses of T-cell cultures. This method was used to produce a panel of 17 protein fractions, each containing two to five protein bands, with a minimal overlap of neighboring fractions (Fig. 2). These fractions were adjusted to the same protein concentration (2 μg/ml) and used to stimu-

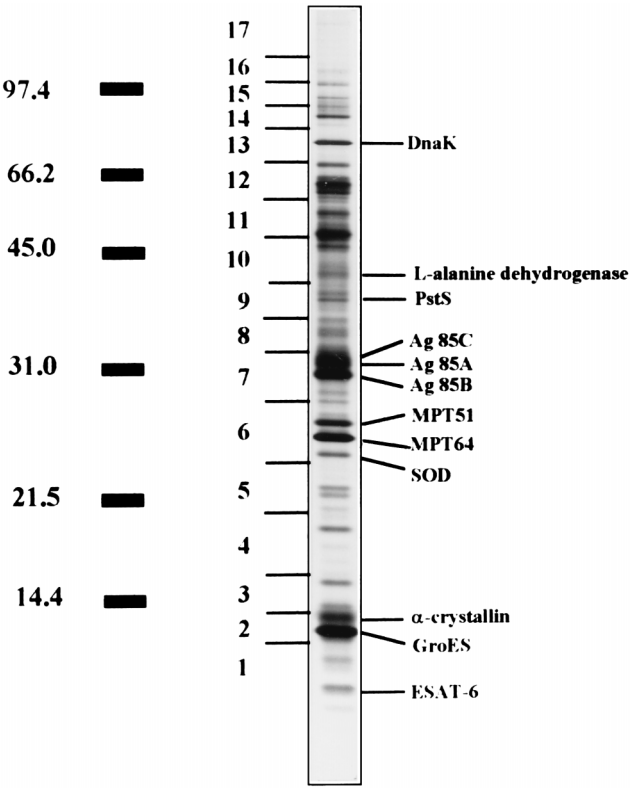

FIG. 2. Culture filtrate proteins in a defined ST-CF harvested from logarithmically growing cultures of *M. tuberculosis*. The filtrate was separated by SDS-PAGE and silver stained. On the left side is shown the molecular mass region covered by each of the 17 protein fractions produced by elution of separated proteins with a whole-gel eluter. The positions of previously characterized culture filtrate proteins identified in ST-CF are indicated on the right.

late PBMC harvested before the onset of infection and at 5 and 16 weeks p.i. Before infection, there was no recognition of the protein fractions (Fig. 3A), but at 5 weeks p.i., a powerful recognition of predominantly low-mass (<14.4-kDa) fractions was induced (Fig. 3B). An average IFN-γ index value of >20 was reached in response to fraction 1 (F1), covering the molecular mass range from 5 to 10 kDa.

The recognition of T-cell antigens at a later stage of experimental tuberculosis infection (16 weeks p.i.) was different. At that time point, all of the fractions induced a significant release of IFN-γ (Fig. 3C). Interestingly, at that stage of infection, although F1 remained an important IFN-γ target, responses to the 16- to 20- and 30-kDa molecular mass regions were of similar magnitudes. This result demonstrated that most of the ST-CF fractions are potentially antigenic but are recognized at different phases of infection. Antigen recognition changes from a highly restricted response that targets low-mass antigens in the early stage of experimental infection to a broad recognition of a range of antigens as the disease progresses.

The low-mass secreted antigen ESAT-6 is a dominant target for IFN-γ-producing T cells in bovine tuberculosis. Recently, a low-mass secreted protein, ESAT-6, expressed in virulent strains within the tuberculosis complex (35) was identified as a target molecule recognized at early time points of infection by immune T cells in a mouse model of tuberculosis (9). To investigate the possibility that this antigen was responsible for the marked responsiveness of infected cattle to low-mass secreted antigens, a comparative test of F1 and purified ESAT-6 was done with PBMC isolated from experimentally infected cattle at 10 weeks p.i. F1 and ESAT-6 stimulated similar levels of IFN-γ in four animals (ODI = 5.5 ± 1.10 and 5.1 ± 1.19, respectively; $P = 0.396$). This result indicated that ESAT-6 is the major target for the bovine T-cell response in the dominant low-mass secreted antigen fraction.

Field cases of bovine tuberculosis are highly responsive to ESAT-6. Cattle from herds with confirmed *M. bovis* infection were studied to determine their recognition of ST-CF antigens. All of the animals investigated had positive skin test responses biased toward PPDB compared with PPDA, indicating ongoing infection with *M. bovis* (Table 1). Preferential T-cell recognition of PPDB compared with PPDA was confirmed in terms of in vitro release of IFN-γ, and the responses to PPDB and ST-CF were of similar magnitudes (Table 1). PBMC from these five animals were stimulated in vitro with the ST-CF molecular mass fractions. All animals exhibited preferential recognition of the low-mass secreted antigens, with IFN-γ responses almost exclusively targeted to antigens of <14.4 kDa (Fig. 4). In most of the animals, very high index values were attained in response to F1 (ODI = 10 to 30). Interestingly, from the results for experimental infection, this characteristic pattern of antigen recognition indicated that all the animals tested were in the early stages of infection.

To investigate the recognition of ESAT-6 in naturally infected tuberculosis field cases, blood samples were obtained from 19 field cases which had skin test reactions biased toward PPDB. The fact that gross tuberculosis lesions were detected in 11 of these animals during the abattoir postmortem examination indicates a high level of infection, as some animals may have small lesions which remain undetected during that procedure. When PBMC were stimulated in vitro, T-cell responsiveness to PPDB was confirmed by very strong IFN-γ responses (Table 2). Importantly, 18 of the animals gave responses to rESAT-6 that were above the background cutoff value of 2.0. In most of the animals, the response to rESAT-6 was at a very high level and correlated positively with the response to PPDB ($r = 0.801$; $P < 0.001$). This confirmed that ESAT-6 was recognized by a heterogeneous population of cattle which had been infected with *M. bovis* in the field.

DISCUSSION

Any rational development of improved vaccines and diagnostic tests for tuberculosis builds upon detailed understanding of the immune responses associated with the disease. Central to that understanding is the identification of mycobacterial antigens recognized by immune T cells during infection. In recent years, interest in extracellular antigens from mycobacteria as key targets for T cells of relevance for protection has been growing (7, 8, 30). Studies of the mouse model of tuberculosis have provided evidence that these antigens are recognized by the immune system at early stages of infection (7), as well as by T cells recruited at the onset of a protective immune response (9). The current understanding is that this early recognition of infected macrophage ensures efficient immune surveillance and early control of the disease. In recent years, this hypothesis has been supported by the demonstration of efficient subunit vaccines based on culture filtrate antigens from several laboratories (4, 20, 25, 34).

In the present study, the use of an experimental model of tuberculosis infection in cattle has pointed out a dynamic change in the antigenic repertoire recognized by T cells during tuberculosis infection. In the early stages of experimental infection, the low-mass ESAT-6 antigen (9, 35) was clearly the dominant IFN-γ target. However, in later stages of infection,

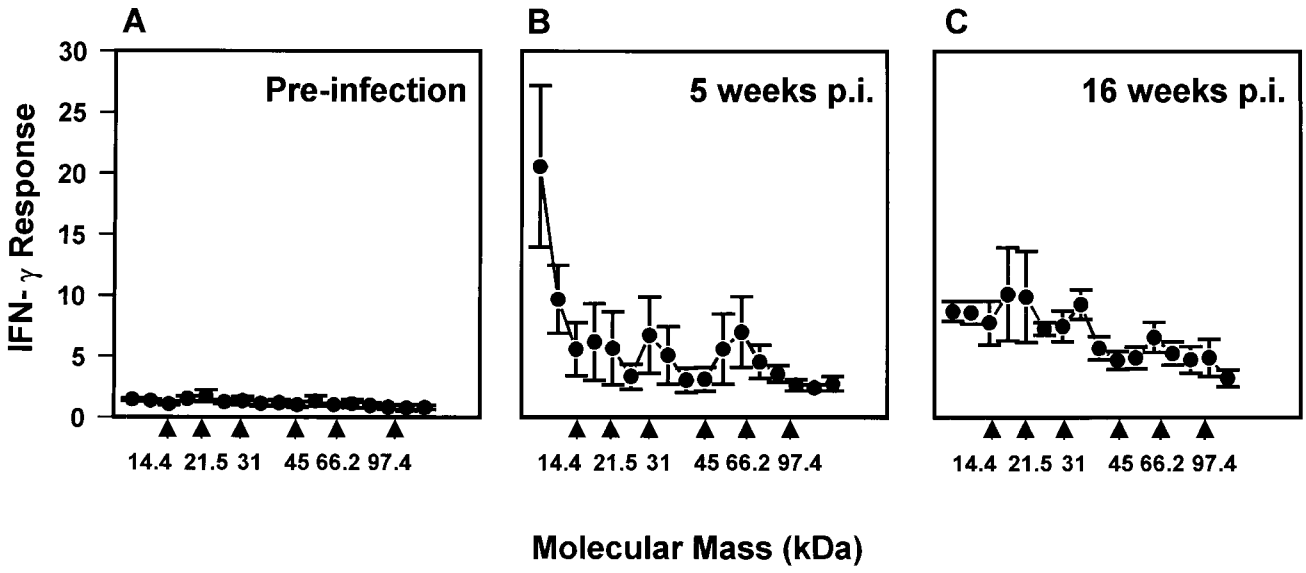

FIG. 3. T-cell recognition of culture filtrate proteins in cattle experimentally infected with *M. bovis*. PBMC were stimulated with molecular mass fractions of ST-CF (Fig. 2), and the release of IFN-γ was measured. The responses given are ODI and have been depicted as means ± standard errors of the means. The locations of molecular mass standards are shown on the *x* axis for comparison. Data were obtained at preinfection ($n = 4$) (A) and at 5 ($n = 4$) (B) and 16 ($n = 3$) weeks p.i.

several other antigens became equally important in the overall IFN-γ response. The highly conserved recognition of low-mass culture filtrate antigens observed in tuberculous cattle is in full agreement with other recent studies of antigen recognition during tuberculosis infection. Lymphocyte proliferative responses induced in the first phase of tuberculosis in the guinea pig model were directed predominantly to low-mass secreted antigens (19), and human patients with active minimal tuberculosis recognized a low-mass antigenic fraction highly enriched with ESAT-6 as the major target for IFN-γ-producing T cells (11). A recent study by Brandt et al. (12) identified two murine T-cell epitopes on the ESAT-6 antigen, and although those epitopes were recognized in the context of different major histocompatibility complex class II molecules, preferential recognition of the ESAT-6 molecule was demonstrated in five of six inbred strains of mice. The limited diversity in the T-cell specificities triggered during the first phase of tuberculosis infection in different species is intriguing; in the present study, this finding was extended by the demonstration that ESAT-6 is a very frequent target molecule in individuals within the same species. This phenomenon is the subject of ongoing research, but it is our current opinion that only a restricted repertoire of mycobacterial proteins may be accessible for recognition by the immune system at these early stages of infection. It is possible that secreted antigens like ESAT-6 belong to a particular protein repertoire that is upregulated in response to the hostile environment of the macrophage, as has been demonstrated elsewhere (1, 23). Future studies of cattle with well characterized ST-CF derived from *M. bovis* may reveal additional unique antigens with potential in the diagnosis of bovine tuberculosis.

It has been reported previously that T cells from experimentally infected tuberculous cattle respond to a wide range of higher-mass antigens (15). In the present study, a similar pat-

TABLE 1. Skin test and in vitro IFN-γ responses of cattle from *M. bovis*-infected herds to PPDA, PPDB, and mycobacterial culture filtrate antigens (ST-CF)

Animal no.	Skin test response[a]		IFN-γ response[b]		
	PPDA	PPDB	PPDA	PPDB	ST-CF
1	2	10	3.9	20.7	26.8
2	2	11	5.6	8.0	7.8
3	4	20	8.8	52.2	24.1
4	3	21	2.3	16.6	17.9
5	2	14	2.2	7.2	41.4

[a] Increase in skin thickness (in millimeters) measured at 72 h after intradermal injection.
[b] IFN-γ responses were measured by ELISA and expressed as ODI.

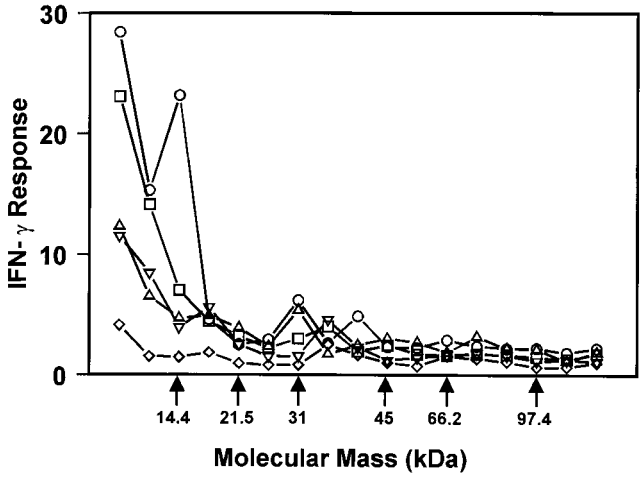

FIG. 4. Recognition of culture filtrate proteins by field cases of bovine tuberculosis. The in vitro T-cell responses of five animals from a herd with confirmed *M. bovis* infection were measured. PBMC were stimulated in vitro with 17 narrow molecular mass fractions derived from ST-CF (Fig. 2), and IFN-γ release was measured and expressed as ODI. The variations between individual wells are not indicated but were generally <5%. The locations of molecular mass standards are shown on the *x* axis.

TABLE 2. Skin test responses to PPDB and in vitro IFN-γ responses to PPDB and rESAT-6 for cattle from *M. bovis*-infected herds

Animal no.	Skin test response[a]	IFN-γ response[b]	
		PPDB	rESAT-6
1	7	29.2	22.8
2	6	13.3	2.4
3	7	14.3	11.1
4	6	19.9	15.7
5	13	22.3	25.0
6	14	18.4	25.3
7	7	18.9	6.7
8	10	17.9	17.2
9	10	25.4	21.3
10	10	5.9	2.9
11	10	11.2	5.8
12	4	7.0	3.1
13	8	15.4	15.6
14	32	14.4	15.5
15	14	17.0	18.1
16	59	10.3	9.8
17	18	13.5	15.3
18	7	8.8	0.8
19	10	8.2	4.4

[a] Increase in skin thickness (in millimeters) measured at 72 h after intradermal injection of PPDB.

[b] IFN-γ responses were measured by ELISA and expressed as ODI. A significant positive correlation was found between responses to PPDB and rESAT-6 ($r = 0.801$; $P < 0.001$).

tern of responses was observed in the more advanced cases of experimental infection. At that point, several of the molecular mass fractions, including F1, gave significant IFN-γ responses. Importantly, however, the pattern of dominant recognition of the low-mass fraction was seen in all the field cases of bovine tuberculosis tested, suggesting that all of those animals were in the first phase of infection. This finding should be related to the ongoing control program for bovine tuberculosis in Northern Ireland. Under this program, all cattle are skin tested annually for the presence of PPD reactors, which are then removed from the herd. This program would therefore be expected to prevent infected animals from reaching later stages of infection. Another important factor in comparing field cases and experimentally infected cattle is the markedly different sizes of the infectious inoculum under the two circumstances. Experimental infection was initiated with an inoculum of approximately 5×10^6 CFU, but natural field cases probably result from a much lower level of infection, possibly as little as a single infectious airborne nucleus (27). This difference obviously influences the length of the lag period before infection gets established. According to this line of reasoning, the high inoculum results in accelerated development of the disease and exposure to a greater range of mycobacterial antigens at earlier stages of infection than those of naturally infected animals.

Recent studies have suggested that a blood test based on in vitro detection of IFN-γ responses may serve as an alternative to skin testing for the diagnosis of tuberculosis in cattle (37) and humans (36). Hence, the identification of dominant IFN-γ targets is of great practical importance for the development of improved tests. Besides being of basic scientific interest, the finding that ESAT-6 is such a conserved target molecule in different individuals therefore suggests that this molecule has potential as a diagnostic reagent. In control programs for bovine tuberculosis, cattle which have skin and in vitro responses biased toward PPDA compared with PPDB are often considered reactive because of exposure to *M. avium* and/or other mycobacterial strains present in the environment. In this regard, ESAT-6 has recently been demonstrated to be absent from almost all nonpathogenic species of mycobacteria outside the tuberculosis complex (18, 35). ESAT-6 may therefore allow differentiation between cattle infected with *M. bovis* and cattle sensitized by environmental strains. A very recent study in which the in vitro responses to ESAT-6 of PPD-reactive cattle from tuberculosis-infected and environmentally sensitized herds were investigated has confirmed the potential of this reagent (31).

In conclusion, the present study has identified ESAT-6 as an important antigen for IFN-γ-producing T cells active in the first phase of tuberculosis infection. This molecule is recognized widely by field cases of bovine tuberculosis, and as ESAT-6 is unique to pathogenic mycobacteria, our findings suggest that this antigen could form the basis of a specific diagnostic test or be a component of a vaccine.

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