Yersinia enterocolitica immunodominant 60 kDa antigen, common to a broad range of bacteria, is a heat-shock protein

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Monoclonal antibodies (mAbs) against the Yersinia enterocolitica immunodominant 60 kDa antigen, termed cross-reacting protein antigen (CRPA), were obtained by fusion of spleen cells from mice immunized with CRPA with murine myeloma cells. The reactivities of the mAbs were examined by Western blotting against extracts of Y. enterocolitica and 23 other species of Gram-positive and Gram-negative bacteria. Cross-reactions were recognized with a wide range of bacteria, but not with Gram-positive cocci. The reactivities were different for each mAb, suggesting that both species-specific and multiple cross-reactive epitopes were present on the CRPA molecule. CRPA was produced under heat-shock conditions in Y. enterocolitica and was shown to correspond immunologically to the GroEL protein in Escherichia coli, a protein involved in the morphogenesis of coliphage. In addition to CRPA, at least nine other major heat-shock proteins were detected by two-dimensional gel electrophoresis of extracts of heat-shocked Y. enterocolitica.

Introduction

A 60 kDa protein antigen is shared by a wide range of bacteria (Ogata et al., 1987). This antigen, designated cross-reacting protein antigen (CRPA), was originally identified as an immunodominant protein which was recognized by IgG antibodies in the sera of patients with yersiniosis and in those of mice experimentally infected with Yersinia enterocolitica (Ogata et al., 1978, 1987). Anti-CRPA IgG antibodies were also detected in sera of healthy humans and the frequency of their detection increased with age (Ogata et al., 1987). By Western blotting analysis, human anti-CRPA sera were shown to react with a 60 kDa protein in crude antigen fractions prepared from Gram-negative bacteria such as Y. enterocolitica, Vibrio cholerae, Pseudomonas aeruginosa, Shigella sonnei, Proteus mirabilis and Klebsiella pneumoniae (Yamaguchi et al., 1986). CRPA has been purified to homogeneity by immuno-affinity chromatography using a specific monoclonal antibody (mAb) (Yamaguchi et al., 1989). It has a native molecular mass of 400–500 kDa with subunits of 60 kDa (Ogata et al., 1987; Yamaguchi et al., 1989). The purified Y. enterocolitica CRPA cross-reacts with rabbit antisera against Y. enterocolitica, V. cholerae, Escherichia coli, Pseudomonas aeruginosa and S. sonnei (Yamaguchi et al., 1989).

CRPA is different from Kunin's enterobacterial common antigen (Kunin, 1963), which is an amino sugar heteropolymer, and from Hofstra's common antigen (Hofstra & Dankert, 1979), which is found in the outer membrane of Gram-negative bacteria (Yamaguchi et al., 1986). However, the 62 kDa antigen originally identified in Pseudomonas has been shown to be an antigen common to Gram-negative bacteria, the so-called common antigen (CA) (Haiby, 1975; Jensen et al., 1985; Sompolinsky et al., 1980). Recently, the 65 kDa protein antigen of Mycobacterium tuberculosis, which is one of the major immunologically active mycobacterial antigens following infection and immunization, was demonstrated to correspond to CA and to the GroEL protein of E. coli, a protein required for the morphogenesis of coliphage (Shinnick et al., 1988). Because of the reactivity of purified CRPA with antisera against Gram-negative bacteria, including P. aeruginosa, a common epitope might be shared between CRPA and CA. In order to define more closely the antigenicity of CRPA and its cross-reactivity with protein antigens found in other bacteria, including the GroEL protein of E. coli, mAbs were produced and characterized in the present study. Since the GroEL protein is a heat-shock protein, the production of CRPA under heat-shock conditions in Y. enterocolitica was also investigated.

Abbreviations: CA, common antigen; CRPA, cross-reacting protein antigen; mAb, monoclonal antibody.
Methods

Bacteria and crude antigen extracts. All bacteria (Table 1) used in this study were from the Department of Microbiology strain collection, Kyorin University School of Medicine. Cultures grown exponentially were harvested and then washed with phosphate-buffered saline (PBS), pH 7.2 (0.14 M-NaCl in 20 mM-sodium phosphate buffer, pH 7.2). Cells were resuspended in PBS and treated with an ultrasonic disintegrator UK-200P (Tomy Seiko Co.) for 20 min at 20 kHz and then centrifuged at 10,000 g for 1 h. The supernatant fluids (antigens) extracts) were hophorized and stored at -20 °C.

Media. Media used were Heart Infusion agar (Wako Co.) containing 10% (v/v) sheep blood for Streptococcus, Chocolate agar containing 10% sheep blood for Neisseria, Haemophilus and Campylobacter, 1% (w/v) Ogawa medium (Eiken Co.) for Mycobacterium, and Heart Infusion broth for other bacteria. M9 minimal medium for labelling of bacteria with [35S]methionine was prepared as described by Maniatis et al. (1982) and was supplemented with 0.3% glucose, 2 μg thiamin ml-1, 0.03 M-MgSO4, 3 mM-MgCl2, 0.1 M-CaCl2, 3 μM-FrCl3, and all L-amino acids except cysteine and methionine.

CRPA and anti-CRPA serum. CRPA was purified from Y. enterocolitica O:3 (strain ZM20) by immuno-affinity chromatography using a mAb (1A4) as previously reported (Yamaguchi et al., 1989). Anti-CRPA serum was obtained from rabbits immunized with purified CRPA from Y. enterocolitica.

GroEL protein and anti-GroEL serum. The purified GroEL protein from E. coli and rabbit anti-GroEL serum were kindly provided by C. Georgopoulos, Department of Cellular, Viral and Molecular Biology, College of Medicine, University of Utah, Salt Lake City, Utah, USA.

Production of mAbs. BALB/c mice were injected intraperitoneally with partially purified CRPA mixed with Freund's complete adjuvant (Difco). Ten days later, the mice were injected intraperitoneally with CRPA suspended in Freund's incomplete adjuvant (Difco). This immunization step was repeated three times at ten-day intervals. Finally, a booster injection was given intravenously using only partially purified CRPA. Three days after the final injection, the spleens of the mice were removed and the spleen cells were fused with murine myeloma cells (P3-X63-Ag8-U1) following the procedure described by Kohler & Milstein (1975). The hybridomas producing apparently specific mAbs against the 60 kDa CRPA were identified by enzyme-linked immunosorbent assay (ELISA) as described by Engvall & Perlmann (1972), and cloned twice by limiting dilution. The mAb preparations for Western blot analysis were culture supernatant fluids of the hybridomas.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. SDS-PAGE (12.5%, w/v, acrylamide) was carried out as described by Laemmli (1970). Crude antigen (25 μg) was loaded in each lane. Western blot analysis was carried out as described by Towbin et al. (1979). After electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) at 0.25 A overnight. After blocking with 3% (w/v) gelatin in Tris-buffered saline (0.15 M-NaCl, 10 mM-Tris/HCl, pH 7.4), the membranes were sequentially treated for 1 h with rabbit anti-CRPA serum (1:1000), rabbit anti-GroEL serum (1:1000) and each mAb (1:10), each diluted in blocking buffer. They were then incubated with goat anti-rabbit IgG (1:500) or goat anti-mouse IgG, IgM and IgA peroxidase conjugate (1:500) (Miles Yeda) for 1 h. Dilutions were made in Tris-buffered saline containing 0.05% Tween 20 (Sigma) and 1% (w/v) BSA (Sigma). Immunoblots were developed with Tris-buffered saline containing H2O2 and o-dianisidine (Sigma).

Two-dimensional gel electrophoresis. Isoelectric focusing in the first dimension (1-6%, v/v, Ampholines pH 5.0-7.0; 0.4%, v/v, Ampholines pH 3.5-10) was performed according to the procedure of O'Farrell (1975) as modified by Georgopoulos et al. (1982). SDS-PAGE in the second dimension was performed as described above.

Heat-shock response. Y. enterocolitica O:3 (strain ZM20) was grown to the mid-exponential phase in M9 medium at 20 °C. Cells were harvested by centrifugation and resuspended at 10^9 cells ml^-1 in M9 medium pre-warmed to the required temperature. Of this suspension, 1 ml each was incubated at either 20 or 42 °C for 10 min, and then labelled with 50 μCi (18:5 MBq) of [35S]methionine for 5 min at the respective temperature. Cells were harvested by centrifugation, and resuspended in 100 μl of lysis buffer [1:5 ml-urea, 0.4 ml-2-mercaptoethanol, 3-2% (v/v) Nonidet P-40, 8% (v/v) Ampholines pH 5.0-7.0, 1-6% (v/v) Ampholines pH 3.0-10], lysed by seven freeze-thaw cycles (frozen at -80 °C and thawed at 37 °C in a water-bath, each for 5 min) and stored at -80 °C.

Results

Species specificity of CRPA mAbs

Screening of hybridoma supernatant fluids for reactivity with the CRPA from Y. enterocolitica (Yamaguchi et al., 1989) yielded four clones. Table 1 shows the reactivities of these mAbs, termed 1A4, 5C1, 5C3 and 3C8, in Western immunoblotting against crude antigen extracts of 24 bacterial species. mAb 3C8, which had the broadest reactivity, recognized a band of 60-65 kDa in crude antigen extracts from 19 other bacterial species comprising Gram-negative rods, Gram-negative cocci and Gram-positive rods. Since this pattern of reactivity was similar to that with anti-CRPA polyclonal antibodies, mAb 3C8 may recognize a framework epitope on the 60 kDa CRPA from Y. enterocolitica, indicating that this mAb recognized a species-specific epitope. The reactivity of mAb 5C1 suggests that this mAb recognized a shared epitope present on CRPA of Y. enterocolitica and its equivalent protein in V. cholerae.

Induction of CRPA under heat-shock conditions in Y. enterocolitica

Y. enterocolitica can grow over a wide range of temperatures (Gronberg & Kihlstrom, 1989). Balanced growth can be sustained even at 4 °C. During the search for optimal conditions for CRPA production prior to protein purification, it was observed that the amount of CRPA in Y. enterocolitica was apparently greater, as judged by Western immunoblotting, in the crude antigen fraction of cells grown at 37 °C or 42 °C rather than at 25 °C, which is the conventional temperature for cultivation of Y. enterocolitica (data not shown). To determine whether the apparently enhanced production
of CRPA at elevated temperatures was based on a heat-shock response, the proteins induced after heat shock in *Y. enterocolitica* were examined. At least ten major proteins were detected in response to heat shock as early as 15 min after the transfer of the cells to 42 °C (Fig. 1a, b). These ten heat-shock proteins ranged in size from 7-5 to 80 kDa. In Western immunoblotting, anti-CRPA polyclonal antibodies recognized two of these heat-shock proteins, which were identical in molecular mass (Fig. 1c, d), suggesting that CRPA was post-translationally modified. CRPA was present in cells grown at 20 °C, but its synthesis was enhanced at 42 °C (Fig. 1a, b). These results demonstrate that CRPA is one of the heat-shock proteins in *Y. enterocolitica*.

In *E. coli*, there is a set of 17 heat shock proteins, one of which is the GroEL protein (Neidhardt et al., 1984; VanBogelen et al., 1987). The fact that CRPA is found in the same relative migratory position in two-dimensional gels as the GroEL protein of *E. coli*, coupled with the fact that the native molecular mass of both proteins is >500 kDa, suggested to us that they might be similar or identical proteins (Hendrix, 1979). To confirm this possibility, the reactivity of the purified GroEL protein from *E. coli* and the purified CRPA from *Y. enterocolitica* with both anti-GroEL and anti-CRPA sera in Western immunoblotting was determined (Fig. 2). The bloting patterns with polyclonal antibodies indicated that the CRPA and GroEL proteins shared common epitopes. The possible divergence of epitopes between the GroEL protein and CRPA was also investigated using the four mAbs produced against CRPA. The broadly reactive mAbs 3C8 and 5C3, but not the more specific mAbs 1A4 and 5C1, recognized epitopes on the GroEL protein (results not shown).

**Discussion**

In the present study, species-specific and cross-reactive epitopes have been demonstrated on the 60 kDa CRPA molecule. The patterns of reactivities of the mAbs with extracts of heterologous bacterial species (Table 1) suggest that CRPA has multiple cross-reactive epitopes shared variously by a wide range of Gram-negative and some Gram-positive bacteria. It may be possible to develop a test for diagnosis of infection with *Y. enterocolitica* based on the identification of its CRPA by mAb 1A4. However, the reactivity of mAb 1A4 with

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**Table 1. Reactivities of bacterial extracts in Western immunoblotting with mAbs against CRPA and rabbit anti-CRPA serum**

<table>
<thead>
<tr>
<th>Bacterial extract</th>
<th>1A4</th>
<th>5C1</th>
<th>5C3</th>
<th>3C8</th>
<th>Rabbit anti-CRPA serum</th>
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<tbody>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Vibrio cholerae</em></td>
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<td>+</td>
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<td>+</td>
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<tr>
<td><em>Serratia marcescens</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td><em>Shigella sonnei</em></td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Proteus mirabilis</em></td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Proteus vulgaris</em></td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Salmonella enteritidis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>-</td>
<td>-</td>
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<td><em>Vibrio alginolyticus</em></td>
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<td>+</td>
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<tr>
<td><em>Vibrio parahaemolyticus</em></td>
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<td>-</td>
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<td>+</td>
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<td><em>Haemophilus influenzae</em></td>
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<td><em>Flavobacterium sp.</em></td>
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<td><em>Mycobacterium tuberculosis</em></td>
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<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td><em>Coagulase-negative Staphylococcus</em></td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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</table>

* + denotes a reacting protein band of 60-65 kDa; – denotes no reacting band in this size range.
Fig. 1. Two-dimensional gel electrophoresis of heat-shocked *Y. enterocolitica* extracts and Western immunoblot analyses with mAb 1A4. Bacterial cells were incubated for 10 min at 20 °C (a, c) or at 42 °C (b, d), and then labelled with [35S]methionine for 5 min at the respective temperature. Following electrophoresis of the extracts, as described in Methods, the separated proteins were transferred to nitrocellulose filters and reacted with mAb 1A4 (c, d). Subsequently, the filters were exposed to Fuji X-ray film (a, b). The open arrow in each gel points to the position of the protein which reacted with the monoclonal antibody. The filled arrows point to the positions of the other heat-shock proteins, whose rates of synthesis are higher in the cells labelled at 42 °C.
CRPA from all Y. enterocolitica serotypes needs to be established. Information is available about several cross-reacting protein antigens in diverse bacterial species, viz. the 62 kDa protein which has been called CA in Pseudomonas aeruginosa (Høiby, 1975; Jensen et al., 1985; Sompolinsky et al., 1980), the 65 kDa mycobacterial protein (Shinnick, 1987), the 60 kDa Borrelia burgdorferi protein (Hansen et al., 1988), Coxiella burnetii protein (Vodkin et al., 1988) and the 60 kDa antigen in Legionella (Pliskyatis et al., 1987). Since the most broadly reactive mAb 3C8 recognized both a 65 kDa protein in M. tuberculosis and a 62 kDa protein in P. aeruginosa (Table 1), it is likely that CRPA is related to CA, although this has to be proven by showing cross-reactivity between CA and CRPA.

In Mycobacterium bovis, the 65 kDa antigen is one of the major immunologically active antigens following infection or immunization and is able to elicit a strong delayed-type hypersensitivity reaction in experimental animals (Bruyn et al., 1987). In addition, it stimulates the proliferation of T cells in immune mice, suggesting that it is likely to be involved in the development of cell-mediated immunity (Kaufmann et al., 1987). In contrast, Willem et al. (1985) suggested that arthritis was induced by a T-cell clone which recognized an epitope on the mycobacterial 65 kDa antigen.

The development of the arthritis-like Reiter's syndrome is often preceded by various bacterial infections, e.g. with Y. enterocolitica, Shigella flexneri, Salmonella typhimurium, K. pneumonias, Campylobacter jejuni and Chlamydia trachomatis (Calin, 1984). Ogasawara et al. (1986) reported that the 60 kDa and 80 kDa antigens of K. pneumonias reacted with anti-HLA-B27 mAb and that lymphocytes from chronic arthritis patients who were HLA-B27-positive reacted with anti-K. pneumonias antibodies. These findings suggest that the broadly conserved bacterial 60–65 kDa antigens such as CA, CRPA and mycobacterial 65 kDa protein may play a role in the development of auto-immune disease which is preceded by bacterial infection.

In the present study, we have shown that CRPA is one of the proteins induced by the heat-shock response in Y. enterocolitica and that it corresponds immunologically to the GroEL protein, a major heat-shock protein in E. coli (Neidhardt et al., 1984). The mycobacterial 65 kDa antigen has also been shown to be related to the GroEL protein (Shinnick et al., 1988). Although the role, if any, of the GroEL protein in the heat-shock response has not been elucidated, it is known to be involved in the morphogenesis of coliphage (Friedman et al., 1984) and also to be essential for E. coli growth (Fayet et al., 1989). Heat-shock proteins are synthesized in response to various environmental stresses including a sudden increase in temperature and are highly conserved among prokaryotic and eukaryotic organisms (Lindquist, 1986; Neidhardt et al., 1984). The possible induction of heat shock in pathogens when a bacterial infection occurs and the consequent release of cross-reacting antigens like CRPA, CA and mycobacterial antigen may result in a strong immune response to these proteins because of possible previous encounters with the same epitopes.

In E. coli, approximately 17 heat-shock proteins are found which are diverse with respect to size, net charge, cellular abundance, and the extents of their inducibilities by heat shock (Neidhardt et al., 1984). Ten of these heat-shock proteins are the products of known genes and have...
been characterized to a greater or lesser extent (Neidhardt & VanBogelen, 1987). We found that ten proteins ranging in size from 7.5 to 80 kDa were induced under heat-shock conditions in *Y. enterocolitica* (Fig. 1).

*Yersinia* spp. harbour plasmids ranging in size from 60 to 75 kb which are necessary for virulence (Gemski et al., 1980; Ben-Gurion & Shafferman, 1981). These plasmids are associated with a number of temperature-inducible features of the bacteria, including production of V and W antigens (Burrows & Bacon, 1960), autoagglutination (Shurnik et al., 1984), and the expression of outer-membrane proteins in the absence of Ca$^{2+}$ (Bolin et al., 1982, 1988). It is possible that these temperature-inducible proteins are virulence attributes for pathogenic *Yersinia* spp.

A detailed analysis of the heat-shock response in *Y. enterocolitica* is required to identify and characterize these gene products.

We thank C. Georgopoulos for providing purified GroEL protein and anti-GroEL antiserum.

**References**


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