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Listeriolysin and IrpA are Major Protein Targets of the Human Humoral Response against *Listeria monocytogenes*

ROLAND GRENNINGLOH,[1]* AYUB DARJI,[1] JÜRGEN WEHLAND,[1]
TRINAD CHAKRABORTY,[2] AND SIEGFRIED WEISS[1]

National Research Center for Biotechnology, Department of Cell Biology and Immunology, D-38124 Braunschweig,[1] and Institut für Medizinische Mikrobiologie, Justus-Liebig Universität Giessen, D-35392 Giessen,[2] Germany

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We have examined the human humoral immune response directed against proteins of *Listeria monocytogenes* in both healthy individuals and listeriosis patients. Two major targets for an antibody response were found in individuals that did not suffer from listeriosis: listeriolysin (Hly) and the recently described internalin-related protein (IrpA). In contrast, the humoral response in listeriosis patients appears to be more heterogeneous and included Hly, IrpA, InlB, and ActA as major targets.

The gram-positive bacterium *Listeria monocytogenes*, which is frequently found in soil and food, is usually harmless to healthy individuals. However, in immunocompromised hosts such as the elderly or in patients undergoing transplantation it can cause a fatal disease resulting in sepsis, meningitis, or encephalitis. Infections during pregnancy can lead to abortion or severe infection of the newborn child (12, 21). An understanding of the immune response mounted against this pathogen is therefore of utmost interest.

In experimental murine listeriosis the interactions between the pathogen and the host have been intensively investigated, and several virulence factors have been characterized functionally and as antigens (4, 14, 22). Following uptake, the bacterium is known to spread from cell to cell (23), thus avoiding contact with the extracellular environment. It is, therefore, protected from the action of antibodies. Accordingly, a protective effect of immune serum on naive hosts has not been found (3, 19). Instead, for the eradication of listeria, cellular defense mechanisms are of crucial importance. These include the action of unspecific cells, such as neutrophils (5, 6) and macrophages, as well as NK cells and specific T cells (14).

Recently, there have been reports on the detection of specific antibodies to listeriolysin in listeriosis patients (1, 9, 11) and in listeria-infected sheep (15, 18). These investigations were carried out in order to develop a sensitive diagnostic assay for infections with *L. monocytogenes*, since these bacteria often cannot be isolated from patients with suspected listeriosis. Similarly, antibodies against p60 have also been detected in listeriosis patients (10) at levels that even surpass those detected against Hly. Antibodies against other unidentified proteins derived from bacterial supernatants were also detected in that study. In addition, Renneberg et al. (20) observed specific antibody formation against an unidentified listerial 93-kDa protein derived from bacterial lysate.

Here we analyzed in detail the proteins which are recognized during the human humoral immune response against *L. monocytogenes*. Healthy individuals who regularly came into contact with *L. monocytogenes* as a result of their profession were chosen for this study. This was done because we intended to study the antibody response under the conditions of a functional immune system, since in contrast, most listeriosis patients are immunocompromised. Additionally, we analyzed antisera from reconvalescent patients that had suffered from listeriosis.

Sera were prepared from healthy donors and stored frozen in aliquots until use. Sera of confirmed listeriosis patients were kindly provided by K. Notermans, Rijksinstituut voor Volksgezondheid, The Netherlands.

Bacteria used in this study were the nonpathogenic strain *Listeria innocua*, the pathogenic strain *L. monocytogenes* EGD pERL50-1 *prfA* 7973, and mutants Δ*hly2* (13), Δ*hly2 prfA* 7973, and *irpA2*. The multicopy plasmid pERL50-1 *prfA* 7973 contains a *prfA* allele from *L. monocytogenes* NCTC7973 and results in overexpression of *prfA*-regulated virulence factors. Listeriolysin was purified according to the procedure described by Darji et al. (7). Internalin B (InlB), IrpA (16), and ActA were purified and kindly provided by S. Müller, A. Lingnau, and J. Wissing, respectively. Bacterial supernatants and cell wall extracts were prepared as previously described (17). The p60 protein was purified from supernatants of *L. monocytogenes* Δ*hly2*, a strain that does not produce listeriolysin. Formic acid (50 mM) was added to the concentrated culture supernatant, and the pH was adjusted to 4.0 with HCl. Batches of 50 ml were applied to a cation exchange Mono S HR5/5 column (Pharmacia, Uppsala, Sweden), and the column was washed with 50 mM Bicine (pH 8.35) and eluted with the same buffer containing 1 M NaCl.

Concentrated supernatants or cell wall extracts of *L. monocytogenes* and *L. innocua* were used as antigens in immunoblots. The blots were incubated with sera of healthy donors. Two major bands with apparent molecular masses of 58 and 30 kDa were stained in both cell wall extracts and supernatants. Some of the sera only recognized one of the proteins strongly, while others detected both (Fig. 1). Only sera from donors with suspected contact to *Listeria* spp. stained these two bands. The bands were not detected in protein preparations of *L. innocua* (data not shown). From the molecular masses of 58 and 30 kDa, we suspected the two proteins to be Hly (7) and IrpA (16), respectively. Therefore, purified Hly or IrpA was tested by immunoblot analysis. Identical to the results displayed in Fig. 1, some of the sera detected only one of the proteins, while others recognized both (Fig. 2A). To confirm the identity of

* Corresponding author. Mailing address: National Research Center for Biotechnology, Department of Cell Biology and Immunology, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49-531-6181-253. Fax: 49-531-6181-292. E-mail: rgr@gbf-braunschweig.de.

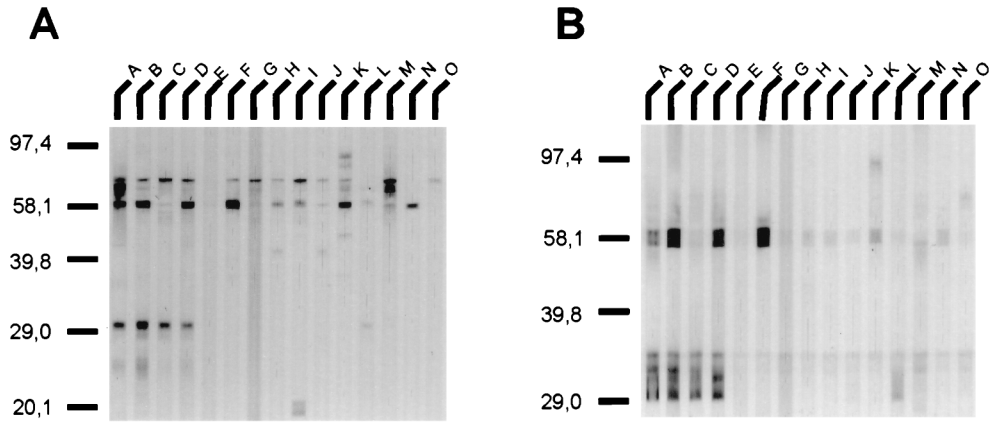

FIG. 1. Two proteins of *L. monocytogenes* are stained in an immunoblot assay by human sera from healthy donors. Cell wall extracts (A) or concentrated supernatants (B) of *L. monocytogenes* EGD pERL50-1 *prfA* 7973 were used as antigens in the immunoblot assay. Letters at top of each panel represent donors and numbers at left are molecular mass markers in kilodaltons.

the two major bands, cell wall and supernatant preparations from *L. monocytogenes* strains were used which no longer produce Hly or IrpA. As expected, no 58-kDa band could be detected when extract or supernatant from an Hly deletion mutant was used, while the 30-kDa band was missing in preparations from the IrpA$^-$ strain (Fig. 2B). This was true for all positive donors tested in this study (data not shown). Additionally, purified InlB and ActA were used as antigens in immunoblots. These two proteins could be stained only with the serum of a single donor (Fig. 2A, donor A; also data not shown). The same serum also recognized InlB in the cell wall preparation of *L. monocytogenes* (Fig. 2B, lanes labeled CW).

The results obtained so far indicated that Hly and IrpA were the main proteinaceous targets of the human antibody response against *L. monocytogenes*. Nevertheless, there have been reports that the murein hydrolase p60, which has an apparent molecular mass of 60 kDa, is an even more potent inducer of an antibody response than Hly in humans (10). Since we were not able to identify a corresponding band with crude protein preparations from the deletion mutant Δ*hly2*, we purified p60 and used it as an antigen in immunoblot experiments. While p60 was recognized by the specific monoclonal antibody IC87C8D10, it was only weakly stained by two of the antisera tested (Fig. 3, donors F and G) and not at all by the others, despite their obvious reactivity towards the listerial proteins Hly and IrpA.

To obtain a quantitative evaluation of the reactivity of the antisera enzyme-linked immunosorbent assays (ELISA) were performed with purified Hly, IrpA, InlB, ActA, and p60 as antigens. Immunoglobulin G (IgG) titers were calculated as reciprocal dilutions of sera which resulted in half-maximal color reactions. Values obtained for Hly, IrpA, and InlB generally corresponded well to the results observed in the immunoblot experiments (Table 1). The highest IgG titers were found against Hly and IrpA. Additionally, sera from donor A showed a high titer against InlB. Again, IgG titers against p60 were generally low, except for sera from donor F which had also shown a weak signal against p60 in the immunoblot assay (Fig. 3). Sera from donors E and G, for whom no contact with *Listeria* spp. was known, did not show a significant IgG response against Hly and IrpA in either assay (Table 1).

The antibodies induced in healthy individuals against listerial proteins could be different from the antibodies induced in patients suffering from listeriosis. Therefore, immunoblot anal-

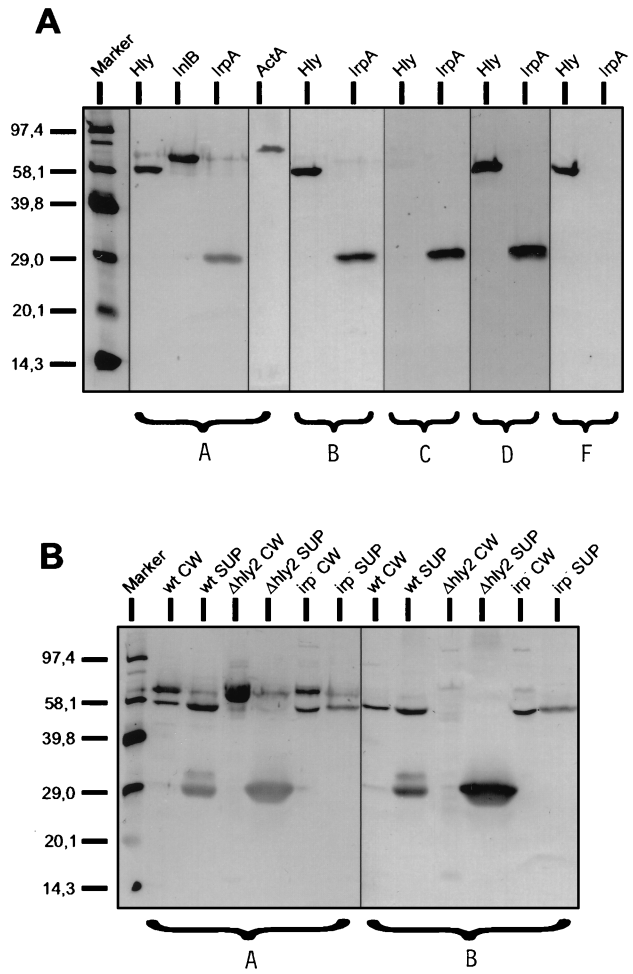

FIG. 2. Identification of Hly and IrpA as the two recognized listerial proteins. (A) Purified Hly and IrpA were used as antigens in immunoblot assays and were stained with sera from different healthy donors as indicated. Only the serum of donor A stained both InlB and ActA. (B) Cell wall (CW) and supernatant (SUP) preparations of *L. monocytogenes* EGD pERL50-1 *prfA* 7973 (wild type [wt]), Δ*hly2 prfA* 7973 (Δ*hly2*), or *irpA2* (irp$^-$) were used as antigens in immunoblot assays and were stained with serum of donors A or B. Vertical lines indicate where the figures have been put together from different immunoblots. Numbers at left are molecular masses in kilodaltons. Letters below panels represent donors.

TABLE 1. Serum IgG titers against purified listerial antigens measured by ELISA compared to reactivity in immunoblots

Subject	IgG titer[a]					Reaction in immunoblot[b]				
	Hly	IrpA	InlB	ActA	p60	Hly	IrpA	InlB	ActA	p60
Healthy donor										
A	1,500	2,000	1,000	20,000	200	++	++	++	+	−
B	200			100	300	−	−	−	−	−
C		500				−	++	−	−	−
D	700			400	800	+	−	−	−	+/−
E	400	1,000		900	300	+	++	−	−	−
F	400	800			300	+	++	−	−	−
G						−	−	−	−	−
H			200	100	400	−	−	−	−	+/−
Listeriosis patient										
1	300	100	300	4,000	400					
2			200	100						
3	3,000	10,000	8,000	5,000	1,000					
4	200	500	400	500	300					
5	500	100		500	500					
6			400							
7	100		100	400	100					
8	100	500	500	400	500					

[a] IgG titers were determined by ELISA. Sera were used at different dilutions. IgG titers are expressed as reciprocal serum dilutions resulting in half-maximal color reactions.

[b] Reactivity in immunoblots was estimated visually. +, positive reaction; ++, very strong reaction; +/−, weak reaction; −, no detectable reaction.

yses were performed with concentrated supernatants of *L. monocytogenes* or mutant strains and developed with sera from eight patients with confirmed listeriosis. As shown in Fig. 4, the pattern of bands recognized by these antisera resembled in general those found with immunoblots with antisera derived from healthy individuals. Seven of eight sera recognized Hly, while four of them (Fig. 4, patients 3, 4, 5, and 8) recognized the 30-kDa IrpA protein clearly. Interestingly, with the other four sera (from patients 1, 2, 6, and 7) a 32-kDa band in the supernatant of *L. monocytogenes* was stained more strongly than the 30-kDa band of IrpA. This 32-kDa band was absent in the IrpA mutant. Monoclonal antibodies recognizing IrpA also cross-reacted with this 32-kDa polypeptide (data not shown). Thus, we believe that it represents an unprocessed or modified form of IrpA. As observed with antisera of healthy donors, no staining of p60 was observed in the immunoblot assay, as there was no 60-kDa band detected in concentrated supernatant preparations of *L. monocytogenes* Δ*hly2* with any of the sera tested. With sera from three patients, an unknown protein with an apparent molecular mass of 40 kDa was detected (Fig. 4, patients 2, 7, and 8). This polypeptide was also lacking in the IrpA insertion mutant. Proteins of similar weight were stained with these sera in supernatant preparations of *L. innocua*, *Listeria seeligeri*, and *Listeria ivanovii* (data not shown).

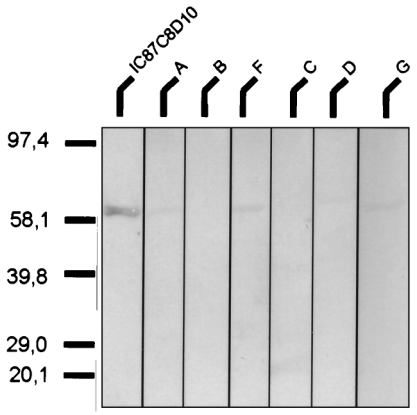

FIG. 3. p60 is not a major target of the human antibody response against *L. monocytogenes*. Purified p60 was used as an antigen in immunoblot assays and was stained either with the specific monoclonal antibody IC87C8D10 (kindly provided by A. Lingnau) or with sera from the indicated donors. p60 was only very weakly stained by sera of donors F and G.

Quantitative analysis of the antibody response with purified proteins confirmed the results obtained by immunoblotting. Generally, in listeriosis patients a broader spectrum of antigens is recognized, compared to that of healthy donors (Table 1). Three patients (no. 2, 6, and 7) had very low IgG titers against all the proteins tested, possibly reflecting an immunocompromised status. Five of eight patients showed significant IgG titers against InlB and six of eight did so against ActA, while in healthy donors significant titers against these proteins were only found in one of five and three of five individuals, respectively, for whom a detectable reaction was observed. Furthermore, in five patients significant IgG titers against p60 were found, while no such antibodies were observed in immunoblot analysis. The recognition of IrpA corresponded very well with the results obtained by immunoblot analysis, but only in three patients could significant IgG titers against Hly be detected by ELISA, while in the immunoblot analysis sera from seven patients showed a clear reaction with this protein. This may be due to a high lipid content in the sera of the patients which may inhibit the recognition of the native protein (7).

Although specific antibodies might play no role in the defense against infection with *L. monocytogenes* (3, 19), definition of antigens detected by sera of listeriosis patients might be of great diagnostic value. Our results clearly show that only two secretory proteins from *L. monocytogenes* induce a strong IgG response in the majority of the healthy donors tested. They were identified as Hly and the recently described IrpA (16), which is identical to InlC (8). IgA antibodies against listerial proteins could not be detected in these donors, excluding the possibility of a permanent colonization (data not shown). The reactivity towards Hly and IrpA was not due to cross-reaction of antibodies against proteins derived from other bacteria,

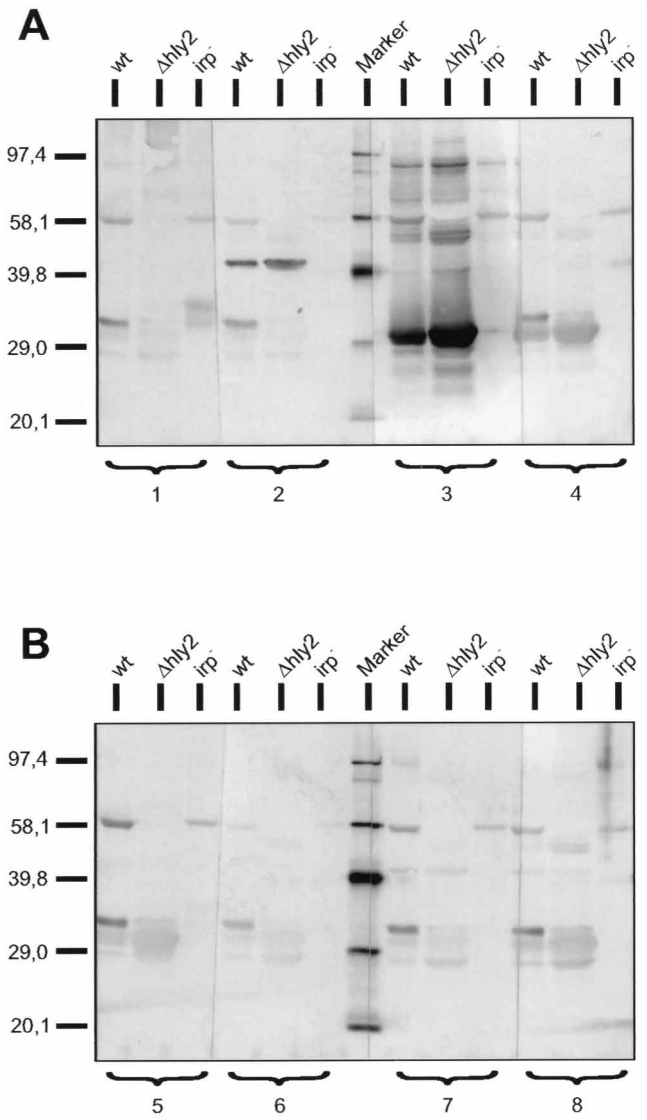

FIG. 4. Sera of listeriosis patients show a similar recognition pattern compared to that of healthy individuals. Concentrated supernatant preparations from *L. monocytogenes* EGD pERL50-1 *prfA* 7973 (wild type [wt]), Δ*hly2 prfA* 7973 (Δ*hly2*), and *irpA2* (irp$^-$) were used as antigens in immunoblot assays and reacted with sera of eight patients (indicated by numbers at the bottom of the gels) with confirmed listeriosis. Sera from most patients recognized listeriolysin O, while sera from four of eight patients recognized the 30-kDa IrpA. Instead, sera from seven of eight patients recognized a 32-kDa band which we believe to be an unprocessed form of IrpA. Additionally, sera from patient 3 recognized a 90-kDa band of unknown origin. Numbers at left are molecular masses in kilodaltons.

since in 20 randomly selected donors no antibodies against these proteins were found (data not shown). The serum of only one of these donors recognized an additional protein, InlB. However, this donor might not be representative as he has been working with this protein including its hyperexpression in *Listeria* spp. Reactivity against two other proteins, ActA and p60, was detected in ELISA using purified proteins but was detected only very weakly or not at all by immunoblotting. In contrast, sera from listeriosis patients contained IgG antibodies against a broad set of antigens, including Hly, IrpA, InlB, ActA, and p60. The reaction towards IrpA differed from that of healthy donors, since in seven patients a 32-kDa protein which we believe to be an unprocessed form of IrpA was recognized strongly, while the 30-kDa IrpA was recognized only by sera of four of eight patients.

Taken together, our data show that Hly and IrpA are major targets of the humoral immune response against *L. monocytogenes* in humans. These antigens, together with InlB and ActA, can be used to probe for and identify listerial infections in patients and, in particular, to assess the incidence of innocuous infection by pathogenic listeria in the general population. The usefulness of p60 as an indicator for infection with pathogenic *L. monocytogenes* is limited. It is found also in nonpathogenic *Listeria* species (2) and only a few donors responded well against this protein. An interesting observation made in this study is that major targets of the humoral response in healthy donors are secreted virulence factors that are generally restricted to pathogenic *Listeria* spp. Listeriolysin is also a major target of the T-cell response in infections, but little or nothing is known about other secreted virulence factors concerning their use as T-cell targets. The significance of these findings is currently being explored by examining a large collection of antisera derived from various sources.

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