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Invasin of *Yersinia pseudotuberculosis* is not a polyclonal activator of mouse B lymphocytes

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ABSTRACT

It is known that the invasin molecule of *Yersinia pseudotuberculosis* stimulates human peripheral B cells *in vitro*. In this work we evaluated the *in vivo* role of invasin as polyclonal activator of B lymphocytes in the mouse experimental model, by comparing strains of *Y. pseudotuberculosis* expressing invasin and isogenic *inv* mutants. Swiss mice were infected intravenously with two strains expressing invasin (YpIII pIB1 and an isogenic virulence plasmid-cured strain, YpIII) and with two invasin mutant strains (Yp100 pIB1 and Yp100, plasmid-cured). Spleen cells were sampled on days 7, 14, 21 and 28 after infection. Immunoglobulin (Ig)-secreting spleen cells were detected by protein A plaque assay and specific antibodies were detected in sera by ELISA. The virulent strain YPIII pIB1 (wild type) did not provoke polyclonal activation of B lymphocytes *in vivo*. In general, fewer Ig-secreting spleen cells of all isotypes were found in the infected animals than in the control animals. Specific IgG antibodies were detected in the sera of animals infected with all strains. The peak response occurred on the 21st day post-infection, and the Yp100 strain provoked the highest level of these antibodies. We concluded that invasin is not a polyclonal activator of murine B cells.

Keywords: *Yersinia pseudotuberculosis*; invasin; polyclonal activation; B lymphocytes; specific antibodies.

INTRODUCTION

The gastrointestinal disorders caused by infection with *Yersinia pseudotuberculosis* and *Y. enterocolitica* are enteritis, lymphadenitis or enterocolitis and sequelae such as reactive arthritis, erythema nodosum, uveitis and septicaemia, can occur (Cover & Aber, 1989; Bottone, 1997). The molecular basis of the sequelae is poorly understood. The outer membrane protein invasin is expressed at low temperature and in the early stationary phase of growth (Revell & Miller, 2000; Nagel et al., 2001), that is under the conditions prevailing e.g. in stored food with which *Yersinia* are ingested by the host. Invasin appears to be particularly important in the early phase of the infection (Pepe & Miller, 1993a; Pepe & Miller, 1993b), promoting the efficient intestinal translocation of the bacteria and colonization of the Peyer's patches (Schulte et al., 2000). *Y. pseudotuberculosis inv* mutants were found to be severely defective in invasion but the LD50 remained unchanged, compared to wild-type bacteria (Han & Miller, 1997).

Invasin protein binds efficiently to the leukocyte integrins of non-activated cells because of its high affinity with the receptor (Leong et al., 1990; Ennis et al., 1993; Arencibia et al., 1997). Upon binding to the integrin VLA-4 (very late antigen), invasin costimulates CD4+ and CD8+ T cells and thus facilitates CD3-mediated proliferative responses of lymphocytes (Brett et al., 1993; Ennis et al., 1993). Moreover, invasin induces lymphocyte motility, chemotaxis and haptotaxis (Arencibia et al., 1997). Another effect of the adherence of *Yersinia* via invasin to lymphoblasts is the induction of MHC (major histocompatibility complex) class I restricted CTL (cytotoxic T lymphocyte) responses (Falgarone et al., 1999). Whether the destruction of cells that are not actually invaded by *Yersinia* helps to eliminate the bacteria or rather is detrimental to the host remains unclear (Falgarone et al., 1999). Invasin is also capable of inducing apoptotic and necrotic cell death in T cells (Arencibia et al., 2002).

Binding of invasin-expressing *Yersinia* to β1-integrin receptors of epithelial cells induces activation of a signal cascade involving Rac1, MAP kinases, activation of the transcription factor NF-kB, and the subsequent production of a variety of cytokines and chemokines, including IL-1, IL-8, MCP-1, GM-CSF and TNF-α (Kampik et al., 2000). The *Yersinia* invasin-triggered inflammatory epithelial cell reaction may lead to the recruitment of phagocytes followed by tissue disruption and this may be part of the strategy of the pathogen to promote its dissemination in the host tissue.

Lundgren et al. (1996) verified that the protein invasin of *Y. pseudotuberculosis* promotes binding of the pathogen to resting human peripheral B cells *via* β_1 integrin receptors (CD 29). B cells responded by expressing several activation markers and by growing. In contrast, T cells did

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not react, even though these cells also express CD29 (Lundgren et al., 1996). One highly speculative hypothesis is that *Yersinia* invasin-mediated polyclonal lymphocyte stimulation may be involved in postinfection immune complications such as reactive arthritis (Arencibia et al, 1997).

The purpose of the present study was to verify whether the invasin of *Y. pseudotuberculosis* provokes polyclonal activation of mouse B lymphocytes *in vivo*.

MATERIALS AND METHODS

Yersinia strains

Two strains of *Yersinia pseudotuberculosis* expressing invasin (the WT strain, YpIII pIB1 and an isogenic virulence plasmid-cured strain, YpIII) and two invasin mutant strains (Yp100 pIB1 and Yp100) were used.

Animals

Female Swiss mice (aged from 6 to 8 weeks) were purchased from CEMIB (Unicamp, Brazil). All mice were kept under specific pathogen-free conditions (positive-pressure cabinet) and provided sterilized food and water *ad libitum*.

LD50 assays (Reed & Muench, 1938)

Groups of 25 mice were infected by the intravenous route with successive 10-fold dilutions of the bacterial suspension (1 to 109 bacteria). The mice were monitored twice daily for a 30-day period. Two independent experiments were performed in duplicate for each strain of *Yersinia*.

Experimental infection of mice

Four groups of 25 mice were inoculated intravenously with 0.2 mL of a suspension of YPIIIpIB1 (1 CFU/mL), YP100pIB1 ($2.5x10^4$ CFU/mL), YPIII ($3.73x10^6$ CFU/mL) or YP100 ($6.67x10^7$ CFU/mL). Groups of 25 uninoculated animals were used as controls. Five mice from each group were bled by heart puncture and their spleens were removed for spleen-cell analysis on the 7th, 14th, 21st and 28th day after infection.

Detection of immunoglobulin-secreting spleen cells

Immunoglobulin-secreting spleen cells were detected by the protein A plaque assay (Bernabé et al., 1981). Rabbit anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were purchased from Serotec (Oxford, England). Each assay was performed in duplicate.

Detection of *Yersinia*-specific antibodies

Yersinia-specific antibodies were detected by ELISA (Medeiros et al., 1991). In brief, all polystyrene plates were coated with cell extract (1.25 μg/mL) obtained from the various strains of *Y. pseudotuberculosis*. Mouse sera were diluted 1/320 for IgG and 1/40 for IgM and IgA. Peroxidase-conjugated goat anti-mouse IgG, IgM or IgA (Sigma) were used to detect bound antibodies. Absorbance was read with a Bio Rad microplate reader, at 492 nm. Samples were assayed in duplicate, and the results were expressed as arithmetic means.

RESULTS

LD_{50} of *Y. pseudotuberculosis* strains

The strains were tested to determine the LD_{50} for mice as a measure of their virulence potentials. Representative results of three experiments are shown in Table 1. Our data indicate that the disruption of *inv* in the strain Yp100pIB1 results in a LD_{50} 10,000-fold higher than that of the wild-type strain (i.e., less virulent). The LD_{50} of strain YPIII, cured of the virulence plasmid, was 1,000,000-fold higher than that of the wild-type (and 48.6-fold less virulent than Yp100pIB1). The strain YP100 was completely avirulent for mice.

Kinetics of immunoglobulin-secreting spleen cells

The virulent strain YPIIIpIB1 (wild type) did not provoke polyclonal activation of B lymphocytes *in vivo*. In general, fewer Ig-secreting cells of all isotypes were present in the spleens of the infected animals than in the spleens of the control animals. The only exception was the IgG3-

Table 1 - LD_{50} doses of wild-type and mutant *Y. pseudotuberculosis* strains.

Strain	Relevant marker	LD50
YPIII (pIB1)	Wild-type	2.6
YP100 (pIB1)	Inv^-	$3.7x10^4$
YPIII	$Plasmid^-$	$1.8x10^6$

secreting cells, which were 3-fold higher in the infected animals, on the 21st day post-infection. The absence of expression of invasin in the isogenic *inv*A mutant Yp100pIB1 (*inv*-) provoked increases in the Ig-secreting cells of the isotypes IgG1 (on the 21st and 28th day post-infection, 4-fold), IgG2a and IgG2b on the 28th day (increases of 7-fold and 3-fold, respectively), and IgA (on the 28th day, 3-fold) (Figure 1).

The animals infected with the plasmid-cured strain *Y. pseudotuberculosis* YpIII (*inv*+), showed numbers of Ig-secreting cells similar to or below those of the control animals, with the exception of IgG3-secreting cells, which showed a 5-fold increase on the 28th day post-infection in relation to the controls, similarly to what occurred in animals infected with the WT strain. The isogenic *inv* mutant Yp100 caused an increase in the number of Ig-secreting cells only for the isotypes IgM and IgA, on the 28th day post-infection (increases of 3.8- and 2.3-fold, respectively) (Figure 2).

Detection of *Yersinia*-specific antibodies

Specific antibodies of the IgG isotype were detected in the sera of animals infected with all strains. The peak response for mice infected with YpIIIpIB1, YpIII and Yp100 occurred on the 21st day post-infection, the Yp100 strain provoking the highest level of these antibodies (Figure 3). In the sera of mice infected with Yp100pIB1, the level of antibodies detected on the 21st day was maintained until the 28th day post-infection (Figure 3).

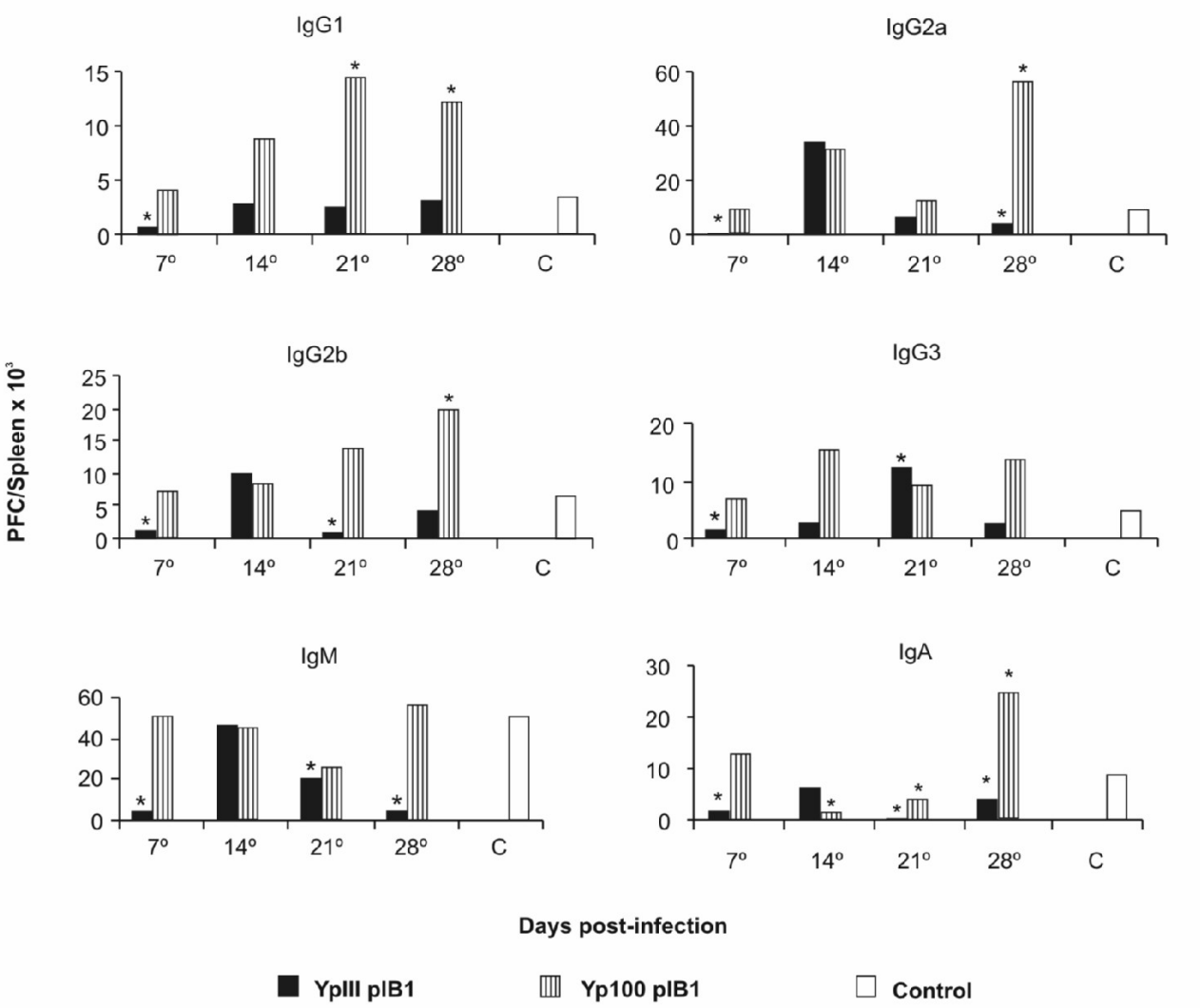

Figure 1. Number of Ig-secreting cells of IgG1, IgG2a, IgG2b, IgG3, IgA and IgM isotypes present in the spleen of mice infected intravenously with YpIII pIB1 and Yp100 pIB1, detected by Protein A plaque assay. Control (C) refers to the average values obtained with spleen cells from uninfected mice throughout the experiment. Results shown are average values of 5 mice in each group. * significantly different from C (Student's *t*-test with $P<0.05$).

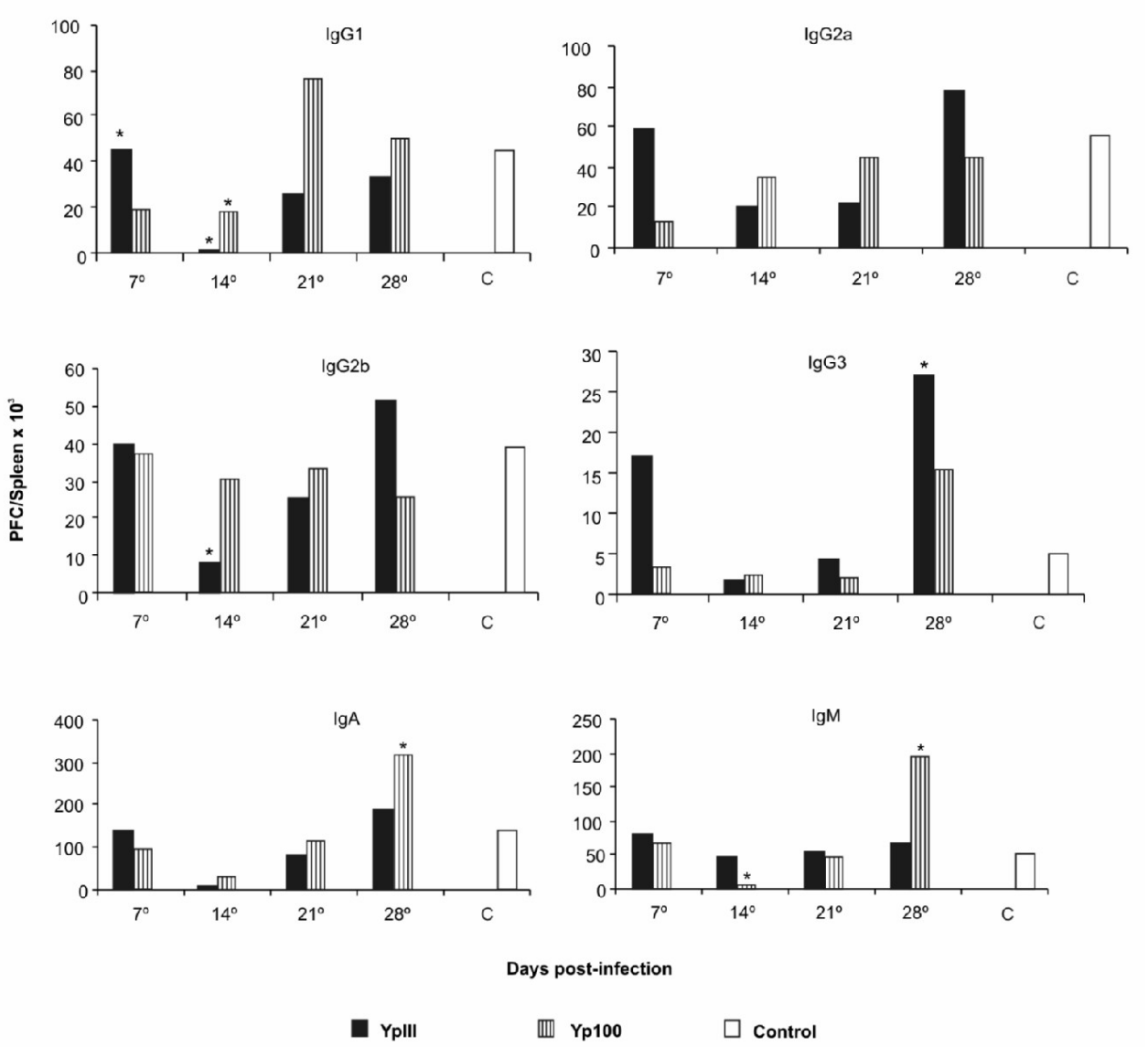

Figure 2. Number of Ig-secreting cells of IgG1, IgG2a, IgG2b, IgG3, IgA and IgM isotypes present in the spleen of mice infected intravenously with YpIII and Yp100, detected by Protein A plaque assay. Control (C) refers to the average values obtained with spleen cells from uninfected mice throughout the experiment. Results shown are average values of 5 mice in each group. * significantly different from C (Student's *t*-test with $P<0.05$).

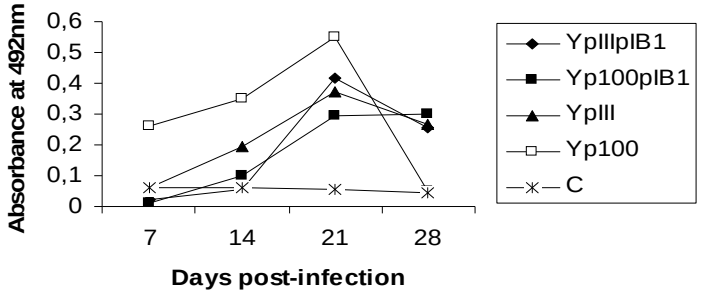

Figure 3. Kinetics of development of IgG anti-*Yersinia* antibodies in the sera of mice inoculated intravenously with YpIII pIB1, Yp100 pIB1, YpIII and Yp100. The sera were diluted 1/320. Control (C) refers to the average values obtained with sera from uninfected mice throughout the experiment. Results shown are average values of 5 mice in each group.

DISCUSSION

The invasin proteins of the two enteropathogenic species *Y. pseudotuberculosis* and *Y. enterocolitica* are highly homologous, except for a homomultimerization domain which is only present in *Y. pseudotuberculosis* (Dersch & Isberg, 1999; Dersch & Isberg, 2000).

The role of invasin in the pathogenesis of *Yersinia* remains obscure. Both the *in vitro* and *in vivo* expression studies suggest that invasin plays an important role in the early phase of intestinal infection (Revell & Miller, 2000, Nagell et al., 2001). The experimental mouse infection model supports this assumption, but at the same time conflicting results have come from different laboratories. An invasin-deficient *Y. enterocolitica* mutant was 80-fold less invasive than the wild-type strain and showed a delay of 3-4 days before it began to colonize Peyer's patches. The LD_{50} (BALB/c mice infected by oral and intraperitoneal route) and the systemic infection phase, however, were unchanged (Pepe & Miller, 1993b; Pepe et al., 1995). In work with *Y. pseudotuberculosis*, Rosqvist et al. (1988) showed that the LD50 of YpIIIpIB1 and Yp100pIB1 were similar (approximately 104 bacteria), while the LD_{50} of the plasmid-cured strain YpIII was 100-fold higher, when Swiss mice were infected by the intraperitoneal route. After oral challenge, however, strain Yp100pIB1 showed a delayed rate of infection, compared to the wild-type strain. Likewise, Han & Miller (1997) showed that *Y. pseudotuberculosis inv* mutants were severely defective in invasion, whereas the LD_{50} also remained unchanged from that of wild-type bacteria. It is, however, obvious from the work of Pepe and Miller (1993a; b) that invasin must have a function in the colonization of Peyer's patches. In this context, it is important to note that invasin is expressed at 37°C after growth of the pathogen in the mouse intestine and that the level of expression is as high as it is for bacteria incubated at 26°C *in vitro* (Pepe et al, 1994). In our study, where Swiss mice were infected by the intravenous route (bypassing the intestinal invasion step), we showed that the disruption of the inv gene in *Y. pseudotuberculosis* (in Yp100pIB1) resulted in a 10,000-fold higher LD50 than that of the wild-type strain (YpIIIpIB1). The strain YPIII (*invA*$^+$), cured of the virulence plasmid, showed a LD50 100-fold higher than the *inv* mutant strain (Yp100pIB1). The strain YP100 (*invA*$^-$, pIB1$^-$) was completely avirulent for mice. These data emphasize the role of invasin in the systemic phase of mouse infection and additionally show that factors encoded by the virulence plasmid pIB1 of *Y. pseudotuberculosis*, such as the Yop proteins, exert a major role in the bacterial pathogenesis.

It has been observed that *Y. pseudotuberculosis* and *Y. enterocolitica* infections are often followed by autoimmune manifestations such as reactive arthritis and erythema nodosum (Toivanen & Toivanen, 1995). The prolonged presence of *Yersinia* antigens has been proposed as the cause of these manifestations (Toivanen, 1994). The increase of autoreactive antibodies resulting from polyclonal B-cell activation might also contribute. The occurrence of polyclonal activation of B lymphocytes in Swiss mice infected with a low-pathogenicity strain, *Y. enterocolitica* serotype O:3, was first demonstrated in our laboratory (Medeiros et al., 1995). Furthermore, Crespo et al. (2002) demonstrated that *Y. enterocolitica* O:3-derived components, such as LPS and Yops, induce polyclonal activation of B lymphocytes. We demonstrated too that infection of mice with either a virulent strain of *Y. enterocolitica* O:8 or with its plasmidless isogenic pair resulted in the polyclonal activation of the splenic B lymphocytes, including some autoreactive clones. (Ramos et al., 2005).

Previous *in vitro* studies from other authors have suggested that invasin molecules might be a source of prolonged immunogenic stimulation, leading to the observed postinfection complications (Lundgren et al., 1996). However, our results proved that this is not the case. We have shown that the *Y. pseudotuberculosis* cell surface-located protein invasin does not provoke polyclonal activation of B cells *in vivo*. These results are surprising and conflict with those obtained previously by Lundgren et al. (1996), who demonstrated that B cells purified from human blood, cultivated in the presence of the virulent strain YpIIIpIB1 (wild type), responded by expressing several activation markers and by growing. The possibility that invasin also triggers differentiation of B cells was not investigated by those authors. We observed a decrease in the number of Ig-secreting cells of all isotypes present in the spleens of mice infected with this virulent bacterial strain, compared with the control animals. On the other hand, we observed an increase in the number of IgG1-, IgG2a- IgG2b- and IgA-secreting cells in the animals infected with the isogenic *inv* mutant Yp100 pIB1 (*inv*$^-$). The plasmid-cured strain (YpIII) showed a behavior similar to that of the wild-type strain. The avirulent strain (Yp100) provoked the increase of IgM- and IgA-secreting cells. Thus, although it has been demonstrated by other authors that invasin is able to activate and induce the growth of human B cells *in vitro*, in the mouse model of infection this protein is not able to induce the polyclonal activation of these cells. On the contrary, *in vivo* the invasin protein of *Y. pseudotuberculosis* exerts an immunosuppressive effect on the mouse B cells.

Yao et al. (1999) demonstrated that *Y. pseudotuberculosis* can directly interfere with the activation of T and B lymphocytes, exerting inhibitory effects on these cells. Primary B cells, transiently exposed to *Y. pseudotuberculosis*, are unable to upregulate surface expression of the costimulatory molecule B7.2 in response to antigenic stimulation. The blocking of lymphocyte activation results from the inhibition of early phosphorylation events of the antigen receptor signaling complex and is dependent on the production of YopH by the bacteria.

An earlier study by our group (Medeiros et al., 2003) demonstrated that Yops isolated from *Y. pseudotuberculosis* did not provoke polyclonal activation of B cells *in vivo* as the Yops of *Y. enterocolitica* O:3 did (Crespo et al., 2002). Sequence comparison showed that YopE, YopH, YopB and YopD are more than 95% conserved among *Yersinia* species

(Michiels et al.,1990). By contrast, some heterogeneity of LcrV was demonstrated among species and strains of *Yersinia* (Roggenkamp et al., 1997). Besides this, YopM can display size polymorphism among the pathogenic *Yersinia* strains (Boland et al., 1998). Ruckdeschel et al. (2001) also demonstrated functional differences among YopP isotypes from diverse pathogenic *Y. enterocolitica* serogroups and YopJ from *Y. pseudotuberculosis*. These differences could explain the diverse immunomodulatory capacities shown by *Y. enterocolitica* and *Y. pseudotuberculosis*.

We conclude that invasin, although an important virulence factor involved in the early phase of intestinal infection, is not a polyclonal activator of murine B cells and might not be involved in the pathogenesis of the immunological sequelae observed in some patients as a complication of *Yersinia* infection.

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RESUMO

Invasina de Yersinia pseudotuberculosis *não é um ativador policlonal de linfócitos B de camundongo.*

A molécula invasina de *Yersinia pseudotuberculosis* estimula células B *in vitro*. Neste trabalho avaliou-se o papel *in vivo* da invasina como um ativador policlonal de linfócitos B, através da utilização de um modelo experimental em camundongos e amostras de *Y. pseudotuberculosis* que expressam invasina e amostras isogênicas mutantes para invasina. Camundongos Swiss foram infectados por via intravenosa com duas amostras de *Y. pseudotuberculosis* que expressavam invasina (YpIII pIB1 e uma amostra isogênica curada do plasmídeo de virulência, YpIII) e com duas amostras mutantes para invasina (Yp100 pIB1 e Yp100, curada do plasmídeo). Células esplênicas foram obtidas no 7º, 14º, 21º e 28º dias após a infecção. Células esplênicas secretoras de imunoglobulinas (Ig) foram detectadas através do ensaio de PFC-Proteína A e anticorpos específicos foram detectados por ELISA. A amostra virulenta YpIII pIB1 (amostra selvagem) não provocou ativação policlonal de linfócitos B *in vivo*. Em geral o número de células esplênicas secretoras de Ig de todos os isotipos, presente nos animais infectados, estava abaixo do número obtido com os animais controles. Anticorpos específicos do isotipo IgG foram detectados no soro dos animais infectados com todas as amostras de *Y. pseudotuberculosis*. O pico de resposta ocorreu no 21º dia após infecção, e a amostra Yp100 provocou o maior nível destes anticorpos. Concluiu-se que invasina não é um ativador policlonal de linfócitos B murinos.

Palavras-chave: *Yersinia pseudotuberculosis*; invasina; ativação policlonal; linfócitos B; anticorpos específicos.

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