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THE O-CHAIN OF BRUCELLA ABORTUS LIPOPOLYSACCHARIDE INDUCES SDS-RESISTANT MHC CLASS II MOLECULES IN MOUSE B CELLS

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Summary. LPS is the most important antigen of *Brucella* bacteria which are gram-negative facultative intracellular pathogens infecting a large proportion of animals and humans in the world. In order to get insights into the immune response mechanisms monitored by *Brucella* , its LPS was used as a model antigen. S-LPS, R-LPS, lipid A and O-chain purified from *Brucella abortus* were tested in their capacity of inducing SDS-resistant MHC class II molecules after incubation with murine B lymphoma cells. S-LPS and O-chain gave a significant response sugesting that O-chain might induce an association with class II itself or might act as a carrier for antigens to bind MHC class II molecules.

Brucella organisms are intracellular parasites infecting a large proportion of humans and animals in the world (1). The means by which these bacteria invade, persist, and reproduce inside cells are not known. However, among factors implicated, the unusual lipopolysaccharide (LPS) seems to play a prominent role. *Brucella* LPS is the most abundant lipid-containing molecule of the *Brucella*. It is exposed to the outer leaflet of the outer membrane where it exists either as high molecular weight complexes or associated with other molecules such as proteins, or other lipid-containing molecules. The chemical structure of the O chain of both *B. abortus* has been elucidated (2,3). It consists of an unbranched linear homopolymer of N-formyl perosamine residues. The O chain is bound to the lipid A by a core oligosaccharide composed of mannose, glucose, quinovosamine, glucosamine, 2-keto-3-deoxyoctonate (KDO) and unidentified sugars. The lipid A moiety possesses both glucosamine and diaminoglucose as backbone sugars, long chain saturated ($C_{16:0}$ to $C_{18:0}$) and hydroxylated (3-OH-$C_{12:0}$ to 29-OH-$C_{30:0}$) fatty acids (3). Ethanolamine, neutral sugars, and ester-linked acyl oxyacyl fatty acids are not found, while phosphate seems to be absent in the lipid A (2,3). In addition, the *Brucella* lipid A possesses a strongly bound group 3 outer membrane protein which is not removed by conventional procedures used to release the lipid A

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associated proteins of enterobacterial LPS (2-8). Although *Brucella* LPS has been implicated in the mechanisms of adherence, penetration and pathogenicity of the bacterial group (9) it is also the most important antigen involved in the immune response of infected individuals (9-11). However, questions concerning the role of different LPS epitopes during the intracellular replication of the bacteria and the immune response of the infected and vaccinated individuals still remain to be answered. Particurlarly, the fate of LPS and LPS moieties (O chain, lipid A and core determinants) in infected cells and their processing (if any) and presentation by infected cells are unknown.

In B cells, class II molecules deal with processed exogenous antigens generated within the endocytic pathway (12). After two minutes of synthesis, class II molecules (α and β chains) are found assembled in the endoplasmic reticulum (ER) (13) in association with the invariant chain (Ii) (14-16). After assembly, $\alpha\beta$Ii complexes are transported out of the ER through the Golgi apparatus. From the trans-Golgi network (TGN), $\alpha\beta$Ii complexes are targeted to the endocytic pathway where they reside at least 2 h before being routed to the cell surface devoid of Ii (12). In murine spleen B cells, the internalization of hen egg lysozyme (HEL) induces the conversion of unstable $\alpha\beta$ class II molecules (U-forms) into stable heterodimers also called compact-forms (C-forms) (17). This stability can be visualized by SDS-PAGE electrophoresis. Indeed, C-forms remain associated in the presence of 1-2% SDS at room temperature (18,19). This stability has been related to class II molecules with bound peptides (20,21). We report here that the purified O-chain of *Brucella* LPS significantly promotes the induction of SDS-resistant class II molecules in a mouse B lymphoma cell line. To our knowledge, this is the first demonstration that an oligosaccharide is specifically involved in antigen-class II molecule binding.

Materials and methods

Antigens. Hen Egg Lysozyme (HEL) from Boehringer Mannheim and Ribonuclease A type IIIA was purchased from Sigma Chemical Co., St. Louis, MO. The bacterial strain Brucella abortus 45/20, its characteristics and the conditions of culture as well as the preparation of LPS derivatives were done according (3,4).

Cell lines. 4E5 B lymphoma cells expressing Ia molecules of the H-2^k haplotype were obtained by fusion between a CH27 B lymphoma subclone (22) sensitive to the HAT selection and B cells purified from CBA/J (H-2^k) mice (23). 4E5 cells were maintained in RPMI-1640 (Gibco-BRL) supplemented with 10% FCS.

Antibody. The mouse anti-I-A^k monoclonal antibody 10.2.16 recognizing both unassembled and assembled forms of I$A\beta^k$ (24,25), was provided from Dr. P. Cosson (Basel Institut, Basel, Switzerland).

Subcellular fractionation. Post nuclear supernatants (PNS) after antigen incubation were obtained as previuously described (26).

Metabolic labeling. 10 x 10^6 cells were used for the preparation of PNS. Cells were pre-cultured for 3 h at 37°C in the presence of 10% fetal calf serum (FCS) supplemented or not with 1 mg/ml of exogenous antigens (HEL, RNase A, S-LPS, R-LPS, Lipid A, O-chain). Cells were washed at 37°C in PBS with 2% FCS, then resuspended in pre-warmed cysteine/methionine-deficient RPMI-1640 medium containing 5% dialyzed FCS (pulse medium). After incubation at 37°C for 1 h, cells were pelleted at room temperature and then resuspended in 3 ml of the pulse medium containing 0.5 mCi of a 80% [^{35}S] methionine and 20% [^{35}S] cysteine mix (Dupont-NEN) and 0.5 mCi of [^{35}S] cysteine (Dupont-NEN). In the pulse-chase experiments with HEL, cells were incubated for 20 min at 37°C. After radiolabeling, cells were incubated for different chase periods in pre-warmed RPMI-1640 medium containing 10% FCS and 5 mM of non radioactive methionine/cysteine (chase medium). In all other conditions, labeling was as above except that the cells were labeled for 12 h at 37°C without chase. Exogenous antigens remained present all along experiments.

Immunoprecipitation. After metabolic labeling, cells were rapidly collected by 5 min centrifugation at 1500 rpm. The PNS was immediately prepared and solubilized for 1 h at 4°C in 1% NP40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF (solubilization buffer). Samples were precleared by a 2 h incubation with a rabbit IgG anti-mouse IgG (Cappel) preadsorbed on protein A-Sepharose CL4B beads (Pharmacia). The immunoadsorbents were discarded after centrifugation (4000 rpm, 5 min using a table-top Eppendorf centrifuge) and the precleared supernatants were incubated overnight with 10.2.16 antibody. Finally, 100 µl of a 50% suspension of protein A-Sepharose beads were incubated with the supernatants for 1 h. Immunoadsorbents were collected by centrifugation, washed three times with 1% NP40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% SDS, 0.1% DOC, 2 mM EDTA, twice with the same buffer without SDS and DOC, twice with 0.5% NP40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA and twice with 10 mM Tris-HCl pH 7.5. The washed beads were resuspended in 60 µl SDS-PAGE sample buffer containing 5 mM DTT. Half of each sample was heated at 95°C for 5 min (boiled: B), the other left at room temperature for 1 h (not boiled: NB). The eluted proteins were then analyzed by SDS-PAGE in 10% gels. Gels were treated with EnHance (Dupont-NEN), dried and submitted to autoradiography. Quantification of radioactivity was obtained by PhosphoImaging (FujiX BAS 1000).

Results and discussion

In mouse splenic cells, it has been first showed by (17) that hen egg lysozyme (HEL) was able to induce SDS-resistant class II molecules (C-forms) when samples were treated in non boiling conditions (NB). We analyzed C-form appearance in the presence or in the absence of HEL in 4E5 B cells in boiling (B) or non boiling (NB) condition. Cells were metabolically labeled for 20 min and chased in the presence (Fig. 1 lower panels) or in the absence of HEL for 0, 1, 2, 4, 8 and 18h.(Fig. 1 upper panels). Class II molecules and associated Ii were immunoprecipitated from the different post-nuclear supernatants (PNSs) with the anti-IA^k monoclonal antibody 10.2.16. When HEL was not present in the cell culture medium, none or very little C-forms were observed under NB conditions (Figs. 1, 3). However, in the presence of HEL, C-forms were detected after 2h-chase and remained after 18h-chase. The formation of C-forms was correlated with the degradation of p31Ii as previously described (17). These results show that 4E5 cells react as much as splenic cells for the formation of C-forms. Quantification of

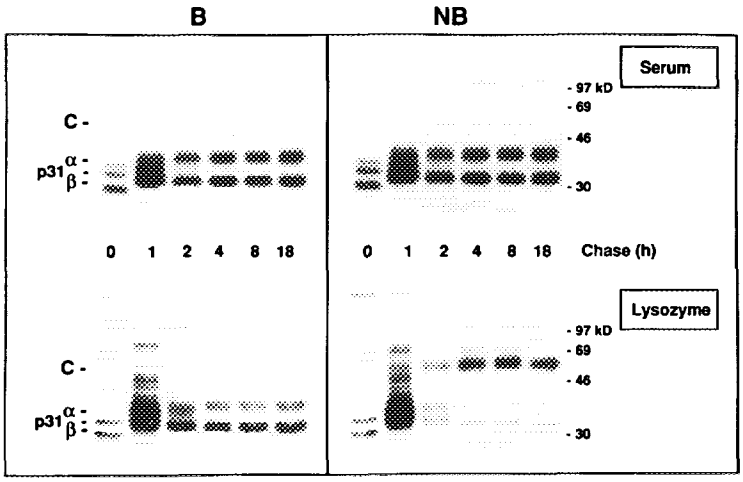

Figure 1. Induction of C-forms by HEL in 4E5 cells.

Distribution of newly synthesized class II molecules and Ii in PNS. 4E5 cells were pre-cultured for 3 h at 37°C with or without 1 mg/ml HEL in the culture medium containing 10% serum. All following steps were carried out in the presence of 10% serum with or without HEL. Cells were pulse-labeled for 20 min at 37°C with 0.5 mCi of a [^{35}S] methionine/cysteine mix and 0.5 mCi of [^{35}S] cysteine and incubated for 0, 1, 2, 4, 8 and 18h-chase time. The PNSs were immediately prepared. Newly synthesized IA^k class II molecules were immunoprecipitated as in Materials and methods and analyzed on a 10% SDS-polyacrylamide gel followed by autoradiography. B : elution with boiling conditions; NB : elution at room temperature; C : C-forms.

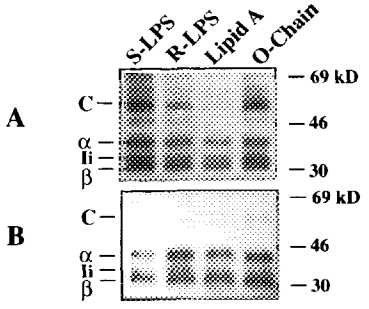

Figure 2. Induction of C-forms by S-LPS, R-LPS, Lipid A and O-chain in 4E5 cells.

Distribution of class II molecules and Ii in PNS. 4E5 cells were pre-cultured for 3 h at 37°C with 1 mg/ml S-LPS, R-LPS, Lipid-A or O-chain in the culture medium containing 10% serum. All following steps were carried out in the presence of 10% serum with LPS antigens. Cells were labeled for 12h at 37°C with 0.5 mCi of a [^{35}S] methionine/cysteine mix and 0.5 mCi of [^{35}S] cysteine. The PNSs were immediately prepared. Newly synthesized IA^k class II molecules were immunoprecipitated as in Materials and methods and analyzed on a 10% SDS-polyacrylamide gel followed by autoradiography. A : elution at room temperature; B: elution with boiling conditions; C : C-forms.

the ratio C-forms/U-forms indicates that HEL C-forms represent 70% of class II molecules detected in the PNS (Fig. 3).

The same approach was used for analyzing the capacity of Brucella antigens to induce C-forms in 4E5 B lymphoma cells. Fig. 2 shows the data concerning the appearance of C-forms in the presence of S-LPS, R-LPS, lipid-A and O-chain under NB and B conditions (Figs. 2A, B, respectively). Although lipid-A does not significantly induce C-forms, S-LPS, O-chain and to a lesser extent R-LPS do. Quantifications in Fig. 3 show that the amount of C-forms generated by S-LPS and O-chain approaches 30% compared to 70% with HEL. This value was found to be similar to that obtained when the cells were incubated with RNase A, another protein presented in the IA^k system (Fig. 3). The amount of C-forms detected with S-LPS and O-chain are significant. Indeed, when using 10% serum or 10% serum supplemented either with lipid-A (Fig. 3) or apamin, a small molecular weight protein not presented in the context of IA^k molecules (not shown), the amount of C-forms detected was less than 7%. Two hypothesis arise from these data. One explanation would be that LPS through its O-chain moiety can bind class II molecules in order to be presented to T-helper CD4+ lymphocytes. In the case of microbial polysaccharides, glycolipids and lipopolysaccharides, the route of internalization, the mechanisms of antigen trafficking, degradation and presentation are practically unknown (27,28). It has been proposed that microbial glycoproteins, lipoproteins, polysaccharides, lipids and glycolipids can be ingested by receptor dependent or independent mechanisms (29) and that they can be detected in different endocytic vacuoles (30). Once inside endosomes, these molecules are susceptible to degradation by both, enzymatic and non enzymatic mechanisms (31-33). In principle, this could be envisioned as a processing mechanism, as in the

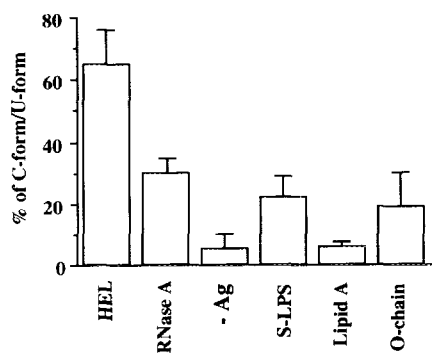

Figure 3. Quantification of % C-forms in the presence of HEL, RNase A and *Brucella* LPS products.

% of C-forms were calculated from the quantification by phosphoImaging of C-form/U-form ratios. Immunoprecipitates obtained from PNSs described in Figures 1 and 2 were analyzed.

case of bacterial peptidoglycan, which is ingested and degraded inside endocytic vacuoles (32). Since the forces which stabilize the protein-peptide interactions are the same forces at work stabilizing protein-carbohydrate and protein-lipid interactions in antibody or enzyme binding, there is no reason to exclude *a priori* that oligosaccharide or lipid moieties could not bind to MHC products or alternative surface molecules for antigen presentation. Since the nominal antigen binding site of class II molecules is capable of fitting molecules from 50 Å to 150 Å long (34), there is the theoretical possibility that non peptide molecules in this size range, such as small oligosaccharides (from 5 to 10 sugars long), would fit the groove of antigen presenting molecules. In addition, the fact that CD 4+ cells could eventually recognize through their T cell receptor, the peptide nominal antigen in the context of MHC II, does not exclude the possibility that non peptide processed antigens associated to MHC II or to other surface molecules, could be recognized by B cells through their Ig receptor. This would indicate that a T-dependent mechanism for antigen presentation is required as suggested by (35-38). Another explanation would be that *Brucella* LPS behaves as a carrier in murine antigen-specific responses (39). In this case, our data indicate that O-chain would mediate and enhance the association of exogenous proteins with class II molecules. Some reports emphasized that the carrier role of *Brucella* LPS would make it as a substitute for the whole bacteria in vaccine development (40,41). Further experiments are required to answer the question of the T-dependent versus T-independent responses induced by *Brucella* LPS; the sytem we developed here represents a key system for investigations at the molecular level of the role of *Brucella* LPS in immune response.

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