CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Sept. 1997, p. 556–564 Vol. 4, No. 5
1071-412X/97/$04.00+0

Humoral Immune Responses of *Brucella*-Infected Cattle, Sheep, and Goats to Eight Purified Recombinant *Brucella* Proteins in an Indirect Enzyme-Linked Immunosorbent Assay

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Received 6 January 1997/Returned for modification 15 April 1997/Accepted 10 June 1997

Brucellosis research is currently focused on the identification of nonlipopolysaccharide (LPS) antigens which could potentially be useful for the specific serologic diagnosis of brucellosis as well as for vaccinal prophylaxis. On the basis of previous reports, we selected eight *Brucella* proteins (OMP36, OMP25, OMP19, OMP16, OMP10, p17, p15, and p39) as candidate antigens to be further evaluated. The genes encoding these proteins were cloned, sequenced, and overexpressed in *Escherichia coli*. The recombinant proteins were purified with a polyhistidine tag and metal chelate affinity chromatography and evaluated in an indirect enzyme-linked immunosorbent assay (iELISA). The specificity of the iELISA was determined with sera from healthy cattle, sheep, and goats and ranged from 95 to 99%, depending on the recombinant antigen and the species tested. Sera from experimentally infected, and from naturally infected, animals were used to evaluate the sensitivity of the iELISA. The antiprotein antibody response was often delayed when compared to the anti-smooth LPS (S-LPS) response and was limited to animals which developed an active brucellosis infection (experimentally infected pregnant animals and sheep and goats from areas where brucellosis is still endemic). Among the recombinant antigens, the three cytoplasmic proteins (p17, p15, and p39) gave the most useful results. More than 80% of the animals positive in S-LPS serology were also positive with one of these cytoplasmic proteins alone or a combination of two of them. None of the recombinant antigens detected experimentally infected nonpregnant cows and sheep or naturally infected cattle. This study is a first step towards the development of a multiprotein diagnostic reagent for brucellosis.

Brucella spp. are small gram-negative bacteria which are facultative intracellular pathogens that produce infectious diseases in humans and animals. Ruminant animals (sheep, goats, and cows) are mostly infected by *Brucella abortus* and *Brucella melitensis* (10).

The classic serologic procedures being used in the diagnosis of ruminant brucellosis (e.g., the complement fixation test, the rose bengal plate test, the seroagglutination test, and the enzyme-linked immunosorbent assay [ELISA]) are mainly based on the detection of antibodies directed against the lipopolysaccharide (LPS) portion of the cell membrane. This dominant antigen is common to both vaccinal and virulent *Brucella* strains. Therefore, distinguishing between the results of infection and vaccination is difficult (15, 35). In addition, tests based on anti-LPS antibodies have extensive cross-reactions with other gram-negative bacteria, which further impair the unambiguous serologic diagnosis of animal brucellosis, especially when the prevalence of the disease is low (9, 24, 27, 33, 35). Among different approaches, the development of diagnostic tests based on antibodies directed to *Brucella* proteins could help to circumvent these problems.

Using either Western blotting (WB) or competitive ELISA (cELISA), several studies have been conducted to analyze the antibody response against proteins of *Brucella* spp. and to identify the immunoreactive proteins which could be used as diagnostic antigens for animal or human brucellosis. Among the immunodominant antigens identified by WB, some belong to the cell envelope and correspond to both major outer membrane proteins (OMPs) (25 to 27 kDa and 36 to 38 kDa) and minor OMPs (10 kDa, 16.5 kDa, 19 kDa, and 89 kDa). With regard to cytoplasmic proteins, a crude LPS-free preparation of cytoplasmic proteins used as an antigen for serologic tests has been shown to allow discrimination between active and inactive human brucellosis (18). By WB, in addition to the heat shock protein, DnaK (6, 38), several immunoreactive bands (39 kDa, 50 kDa, and 20 kDa) were identified in cytoplasmic extracts (36). cELISA targeting selected cytoplasmic (p17) (23), periplasmic (BP26) (28), or membrane (6, 38) proteins has also been described. These experiments led to the conclusions that the antiprotein antibody response was heterogeneous among infected animals and that only a combination of selected *Brucella* proteins could lead to a satisfactory diagnostic test (23, 25, 29).

Analyses such as those described above require the use of *Brucella* proteins in isolated form. Up to now, few serologic studies have been conducted on purified *Brucella* proteins. Among these are cytoplasmic antigens: the Cu-Zn superoxide dismutase (29), an 18-kDa protein (19), and an antigen called A2 (37), which could correspond to the *Brucella* bacterioferritin (15), as well as purified membrane proteins (OMP89 [25] and the peptidoglycan-linked lipoprotein [20]). Recently, a protein useful for the serologic diagnosis of brucellosis was cloned independently by two groups and described either as an OMP belonging to *Brucella* group 3 antigen (26) or as a periplasmic protein (BP26) (28).

In general, these studies involved analysis of the humoral response of a limited number of animals, mostly naturally

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infected and belonging to only one species. A serologic follow-up aimed at analysis of the antiprotein immune response throughout the course of an infection has been very rarely described. Except for WB experiments, most reports deal with only one antigen. These approaches preclude any comparative analysis of the reactivity of different proteins with the same sera, and they also do not allow evaluation of their complementarity. In addition, while specificity is of major concern in brucellosis diagnosis, this aspect was only marginally considered when a small number of negative sera were used and never with sera from animals experimentally infected with bacterial species known to induce immunological cross-reactions with *Brucella* spp.

In order to specifically address these topics, we selected eight *Brucella* proteins (OMP10, OMP16, OMP19, OMP25, OMP36, p15, p17, and p39) as candidate antigens to be evaluated further. These proteins were produced in recombinant form and purified before being used as the antigens in indirect ELISA (iELISA). We report here the systematic evaluation and comparison of the humoral response against these purified recombinant *Brucella* proteins by using sera from naturally or experimentally *Brucella*-infected cattle, sheep, and goats. The specificity of the tests with these recombinant proteins was also analyzed by using sera from healthy ruminants as well as sera from cattle experimentally infected with bacteria known to induce immunological cross-reactions with *Brucella* spp.

MATERIALS AND METHODS

MAbs and antiserum. Anti-OMP10 monoclonal antibodies (MAbs) (A68/8E7/B11 and A68/7G11/C10) were defined as reacting with a 10-kDa band on immunoblots of *B. melitensis* B115 cell extracts; anti-OMP19 (A76/2A4/A7, A68/25H10/A5, A76/17B9/A2, A76/18B2/D6, A76/12F2/D6, A76/5C10/A8, and A76/10D3/H2), anti-OMP16 (A68/4G1/C6, A66/4E3/B7, and A76/8C3/G3), anti-p17 (A66/5H1/E9 and A68/29E3/C10), anti-OMP25 (A76/12H9/A5, A76/8H9/A2, A68/7D11/B3, A59/1E11/D11, A68/4B10/F5, A66/1C6/E1, A70/6B5/A7, A18/13D2/F5, A68/28G6/C7, A76/2C12/C11, A76/2F4/F1, A19/12B10/F4, and A76/15H7/E7), and anti-OMP36 (A63/13G2/C4, A63/5A7/A8, A63/8D8/C7, A63/3H2/B1, A63/15E5/B11, A63/4D11/G1, A68/25G5/A5, A68/15B6/C8, and A63/4F7/E3) MAbs react, respectively, with a 19-kDa, a 16.5-kDa, a 17-kDa, a 25-kDa, or a 36-kDa band under the same conditions (4, 5, 8, 23, 30, 31). Production of MAbs has been described previously (4, 5, 7, 8, 11, 23). Except for the anti-p17 MAbs, all of the MAbs recognize their respective targets on the surfaces of intact *Brucella* sp. cells (4, 5, 7, 8). The exact localization of the p17 protein remains to be determined, but it could be cytoplasmic, based on the sequence identity with an 18-kDa protein described by Goldbaum et al. (19). Anti-p39 MAbs (A43/5E1/E8, A43/5G2/B10, and A43/5G2/E4) react with a 39-kDa band on immunoblots of a crude preparation of cytoplasmic proteins from *B. melitensis* B115 (11), available commercially as Brucellergène (Rhone-Mérieux, Lyon, France). Anti-p15 MAb was commercially available (AMD Ltd., Artarmon, Australia) (21) and proved to react with a 14-kDa band on immunoblots of *B. melitensis* B115 cell extracts. MAbs were used as supernatants of hybridoma cultures. MAbs reactive with the murine tumor necrosis factor (mTNF) peptidic tag or the Cro-LacI leader peptidic tag were produced by Innogenetics. The rabbit antiserum raised to *Escherichia coli* proteins was obtained from Dako (Glostrup, Denmark).

***Brucella* genes.** The cloning and sequencing of most *Brucella* genes have been previously described (11–13, 16, 23, 30, 31).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (31).

Expression and purification of the *B. abortus* recombinant proteins in *E. coli*. The expression and purification of rOMP19 and rOMP10 have been previously described (31). The remaining six recombinant proteins were expressed with two different vectors.

(i) Plasmid construction. The vector pmTNFMPH (17) and its derivative, pIGFH10, allow expression of cloned genes as fusion proteins with a peptide tag comprising the first 25 amino acids of mTNF followed by a hexahistidine peptide. The gene fragment containing the p17 open reading frame (ORF) was isolated by PCR and cloned into pIGFH10 to obtain an in-frame fusion with the mTNF fusion partner. A 454-bp *Brucella* DNA fragment encoding OMP16, devoid of the putative signal peptide, was cloned into pmTNFMPH, again fusing the *Brucella* ORF in frame to the mTNF tag ORF.

The vector pIGALMPH, used for the expression of the other four recombinant proteins, has been described by Van Gelder et al. (32). The fusion proteins synthesized contain 48 amino acids of Cro-LacI followed by a stretch of six histidyl residues at the amino terminus. A 911-bp *Nco*I-*Xba*I DNA fragment encoding OMP36, devoid of the putative signal peptide and of the first 37 amino acids of the mature protein, was subcloned into pIGALMPH. The gene encoding OMP25, devoid of the putative signal peptide, was amplified by PCR and introduced into pIGALMPH in frame with the vector-encoded leader peptide. A 1,364-bp *Bgl*II-*Xba*I DNA fragment containing the p39 coding sequence, devoid of the first 15 codons, was inserted into pIGALMPH. A 450-bp *Fok*I-*Ava*II DNA fragment encoding the protein p15, devoid of the first five amino acids, was cloned into pIGALMPH.

TABLE 1. Characteristics of the recombinant *Brucella* proteins tested

Protein	Fusion partner	No. of amino acids missing[a]	Molecular mass (kDa)[b]	Purity[c] (%)	Solubility[d]	Yield (mg/liter)
OMP10	mTNF	5	14.8	>95	I	10
OMP16	mTNF	0	20.2	>99	S	40
OMP19	Cro-LacI	4	22	>95	I	4
OMP25	Cro-LacI	0	27.4	>90	I	100
OMP36	Cro-LacI	37	39.4	>90	I	100
p17	mTNF	0	21.5	>99	I	23
p15	Cro-LacI	15	17.5	>95	I	7
p39	Cro-LacI	5	46.1	>99	I	35

[a] Without taking into account the signal peptide when relevant.
[b] Calculated molecular mass of the fusion protein.
[c] Estimated by WB with an anti-*E. coli* rabbit antiserum.
[d] I, insoluble (inclusion bodies); S, soluble.

The characteristics of the recombinant proteins are summarized in Table 1.

Fusion proteins produced by these vectors can be detected with monoclonal antibodies directed to the mTNF or to the Cro-LacI tag sequences, respectively.

(ii) Expression and purification of the recombinant proteins in *E. coli*. *E. coli* SG4044 (22), containing plasmid pAc1, was transformed with the relevant expression vector. A 15-liter fermentor was seeded with an overnight saturated culture of the transformants in a 1/50 dilution. Incubation was performed at 28°C until the optical density (OD) at 600 nm reached 0.2. The temperature of the vessel was then increased to 42°C, and incubation continued for an additional 3 to 4 h. During the fermentation, the pH (6.9), dissolved oxygen (50%), and temperature were monitored and adjusted. The bacteria were then concentrated by microfiltration and centrifugation and kept frozen at −70°C until use. After thawing, the cells were resuspended in 150 ml (5 pellet volumes) of lysis buffer (150 mM KCl, 10 mM Tris [pH 6.8], 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 25 mM ε-aminocaproic acid) and lysed in a French press (two cycles at 16,000 lb/in^2). The resulting lysate was centrifuged at 30,000 × *g* for 20 min at 4°C. The pellet was solubilized in 80 ml of 6 M guanidine-HCl–0.1 M phosphate (pH 6.5) buffer containing 0.05% Triton X-100, and the extract was centrifuged at 30,000 × *g* for 30 min at 4°C. The supernatant containing the recombinant antigen (50 to 100 mg of total proteins) was then loaded onto a 20-ml immobilized metal ion adsorption chromatography column (Pharmacia P-L Biochemicals) preactivated with $NiCl_2$ and equilibrated with 6 M guanidine-HCl–0.1 M phosphate (pH 6.5) buffer. The column was washed with 6 M guanidine-HCl–0.1 M phosphate (pH 6.5) buffer containing 0.05% Triton X-100 and eluted with a linear pH gradient (pH 6.5 to 3.7) in the same buffer. The purified proteins were aliquoted and stored at −70°C until use. The protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, Ill.), using bovine serum albumin as the standard. Expression and purification of the recombinant antigens were monitored by SDS-PAGE followed by Coomassie brilliant blue staining or WB analyses. An anti-*E. coli* rabbit serum was used to estimate the level of *E. coli* proteins contaminating the purified recombinant antigens. The reactivity with relevant anti-*Brucella* protein MAbs and with the anti-Cro-LacI or anti-mTNF MAbs was also analyzed by immunoblotting and by iELISA.

Sera. (i) Naturally infected animals. Sera collected from 120 sheep from flocks in which *B. melitensis* had been isolated previously and no vaccination had been performed were obtained from J. M. Blasco (Servicio de Investigacion Agraria, Zaragoza, Spain) (3). These sera were grouped according to the results of bacteriologic, serologic, and delayed-type hypersensitivity (DTH) reactions. Group A consisted of 50 sheep from which *B. melitensis* (biotypes 1 and 3) was isolated. All of these animals gave positive DTH reactions, and their sera were positive for anti-*Brucella* antibodies as measured by the complement fixation test and the rose bengal test. Group B consisted of 50 sheep which were bacteriologically negative but positive for *Brucella* antibodies in classic serologic tests. All of these animals were also positive by intradermal skin testing. Group C comprised 20 animals which were negative in both bacteriologic and serologic tests but still gave a positive DTH reaction. Group D consisted of 15 animals which were negative in bacteriologic, serologic, and skin (DTH) tests. In addition, serum samples from 36 naturally *B. abortus*-infected cattle with positive smooth

LPS (S-LPS) ELISA and positive serologic tests were used. Fifty-four sera from field-infected goats with positive serologic tests were provided by J. M. Blasco and were also tested.

(ii) Experimentally infected animals. Experimental *Brucella* infections in all species were initiated via the conjunctival route. Ten nonpregnant ewes were infected by exposure to 10^5 CFU of *B. melitensis* H38 and were bled every 2 weeks for 22 weeks. The sera were provided by G. Dubray (Institut National de la Recherche Agronomique-Tours, Nouzilly, France). Pregnant goats ($n = 12$) were infected with 5×10^5 CFU of *B. melitensis* H38 and were bled weekly for 2 months. These sera were provided by A. MacMillan (Central Veterinary Laboratory, Weybridge, United Kingdom). These ovine and caprine sera were collected during a European Economic Community (EEC) research program (CAMAR 8001-CT90-0018). Twenty-four heifers were infected with 16.6×10^6 viable cells of *B. abortus* 544 (biovar 1) during the fifth month of gestation; all animals aborted. These animals were bled every 2 weeks for 176 days after the infection. In addition, two groups of four nonpregnant heifers were infected with two different doses of *B. abortus* 544 (1.4×10^7 and 1.4×10^{10} CFU) and were bled weekly for 100 days.

(iii) Positive control sera. The positive control sheep sera for the ELISAs were produced by intramuscular injection of the purified recombinant proteins at 150 μg in saponin QuilA adjuvant (Spikoside; Iscotec Products, Uppsala, Sweden) (1 mg in phosphate-buffered saline [PBS] in a final volume of 2 ml) three times each at 3-week intervals. Preinoculation samples served as the negative control sera; positive control sera were collected 1 week after the last injection.

(iv) Negative sera. Sera from 88 unvaccinated sheep from a *Brucella*-free experimental flock (provided by G. Dubray), 96 serum samples from healthy cattle from a certified brucellosis-free region, and 23 negative goat sera collected from unvaccinated healthy goats (provided by B. Garin-Bastuji, Centre National d'Etudes Vétérinaires et Alimentaires, Paris, France) were used as negative controls. These *Brucella*-negative sera were used to calculate the cutoff of the iELISA performed on the recombinant proteins. The cutoff was determined as the mean of the OD value of the negative sera plus three standard deviations (SDs).

(v) Sera from animals experimentally infected with *Yersinia enterocolitica* O:9. Four heifers were infected per os with 10^{12} viable cells of *Y. enterocolitica* O:9; the procedure followed has been described previously (34).

ELISA procedure. (i) Ovine, caprine, and bovine sera were assayed by iELISA for antibody reactivity against the recombinant proteins as follows. Microtiter plates (MaxiSorp; Nunc A/S, Roskilde, Denmark) were coated with recombinant protein (100 μl per well) in 0.1 M $NaHCO_3$ (pH 9.5) and incubated for 1 h at 37°C. For each protein the optimal coating concentration was determined by testing a wide range of concentrations (0.01 to 10 μg/ml). All recombinant proteins were used at 1 μg/ml except for p15 and p17, which were used at 2 and 3 μg/ml, respectively. When a combination of antigens was used, each individual protein was coated at its own optimal concentration. Wells were saturated with blocking buffer (PBS containing 0.1% caseine and 0.5 mg/ml of octhilinone [Kathon CG; Rohm and Haas, Philadelphia, Pa.]) for 1 h at 37°C. Plates were used immediately after preparation. Sera were diluted 50-fold in blocking buffer containing 0.3% Triton X-705 and *E. coli* lysate to a final protein concentration of 0.5 mg/ml (to reduce interference of anti-*E. coli* antibodies present in the sample sera) and tested in duplicate wells. Binding of antibodies to the recombinant antigens was visualized by using polyclonal peroxidase-conjugated rabbit anti-sheep, anti-goat, or anti-cow immunoglobulin (Dako) diluted 5,000-fold in blocking buffer. Washings between the different incubations and development of the peroxidase activity were performed as described previously (6). The difference in ODs at 490 and at 630 nm was read in a Bio Kinetics reader EL340 (Bio-tek Instruments, Winooski, Vt.). Positive and negative control sheep sera were included in all plates.

(ii) *Brucella* S-LPS prepared as described previously (31) was coated on microplates (69620; Nunc) at 1 μg/ml in carbonate buffer, pH 9.5. The subsequent steps were done as for the protein ELISA.

RESULTS

Expression and purification of the recombinant antigens. The expression and purification of rOMP10 and rOMP19 have been described previously (31).

The putative signal peptides of the different OMPs were removed in order to avoid the processing of this sequence in *E. coli* (31) and the subsequent loss of the polyhistidine-peptidic tag. In addition, this procedure should enhance the expression level and limit the problem of toxicity caused by membrane localization of the expressed OMP. The features of the constructs used to express the *Brucella* proteins in *E. coli* are summarized in Table 1. SDS-PAGE analysis of lysates of *E. coli* transformed with the relevant expression vectors demonstrated that the proteins were readily produced upon induction (Fig. 1A). In most cases, the expression product appeared as a major band upon Coomassie blue staining. The size of this band corresponds to the calculated molecular mass of the fusion products (Table 1) as illustrated in Fig. 1A for the 17-kDa cytoplasmic protein (p17). In addition, a band of similar size was identified after WB of the same extract, using either anti-*Brucella* protein MAbs or MAbs against the fusion peptide to develop the blot (not shown).

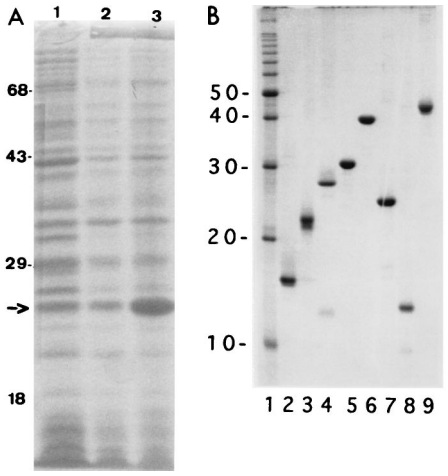

FIG. 1. (A) SDS-PAGE analysis of total cell lysate of *E. coli* producing the mTNF p17 fusion protein. Lane 1, culture grown at 28°C (uninduced); lanes 2 and 3, culture after 1- and 2-h induction, respectively (42°C). The arrowhead indicates the position of the *Brucella* fusion protein. (B) SDS-PAGE analysis of purified recombinant *Brucella* proteins. Lane 1, molecular mass markers; lane 2, OMP10; lane 3, OMP16; lane 4, OMP19; lane 5, OMP25; lane 6, OMP36; lane 7, p17; lane 8, p15; and lane 9, p39. Calibration of the gels is indicated in kilodaltons. Protein detection was by Coomassie blue staining.

Except for rOMP16, which was detected partially in the soluble fraction of the *E. coli* extract, all other recombinant *Brucella* proteins accumulated as insoluble aggregates in *E. coli* (data not shown). The aggregates were solubilized in 6 M guanidine-HCl (or, in the case of rOMP16, supernatant was mixed with solid guanidine-HCl to obtain a final concentration of 6 M) and were purified on a nickel chelate affinity chromatography column. The purity of the recombinant proteins is illustrated in Fig. 1B. This procedure yielded highly purified recombinant proteins in amounts ranging from 4 to 100 mg/liter of culture (Table 1).

Analysis of the antigenicity of the recombinant proteins. The antigenicity of the recombinant proteins was compared to that of the native proteins by using a panel of MAbs in an iELISA.

Two methods of preparing the recombinant proteins for use in the iELISAs were evaluated. The antigens were either diluted directly from the denaturation buffer to the coating buffer or were first dialyzed against PBS and precipitated with cold acetone-methanol before being resuspended in PBS by sonication, and then they were diluted in the coating buffer. The three minor OMPs and the three cytoplasmic proteins remained mostly soluble after resuspension in PBS, while the two major OMPs could never be solubilized by this procedure.

No significant differences in reactivity with relevant MAbs were demonstrated between these two methods of antigen preparation (data not shown). Therefore, for all subsequent tests, the recombinant antigens were directly diluted from 6 M guanidine-HCl solution. All MAbs specific for OMP10 (two MAbs), OMP16 (three MAbs), OMP19 (seven MAbs), p15 (one MAb), p39 (three MAbs), and p17 (two MAbs) reacted in iELISA on the relevant recombinant protein. Therefore, it can be concluded that as far as the epitopes recognized by these

TABLE 2. Summary of cutoff values and specificities of iELISAs with different recombinant proteins

Protein	Negative bovine sera (n = 96)			Negative ovine sera (n = 88)			Negative caprine sera (n = 23)		
	Cutoff[a] (OD)	No. positive[b]	Specificity (%)[c]	Cutoff (OD)	No. positive	Specificity (%)	Cutoff (OD)	No. positive	Specificity (%)
OMP10	0.133	3	97	0.431	2	97	0.240	1	95
OMP16	0.210	3	97	0.354	1	99	0.224	0	100
OMP19	0.131	3	97	0.246	3	96	0.133	1	95
OMP25	0.114	5	95	0.233	2	97	0.207	0	100
OMP36	0.108	4	96	0.174	1	99	0.132	0	100
p15	0.111	4	96	0.209	1	99	0.156	0	100
p39	0.254	2	98	0.196	1	99	0.203	1	95
p17	0.160	1	99	1.141	1	99	0.185	0	100

[a] Cutoff value calculated as the mean of the OD of the negative sera plus three SDs.
[b] Number of positive animals (OD > cutoff).
[c] Calculated specificity, assuming that all the animals were brucellosis free.

MAbs are concerned, the antigenicity of the recombinant products seemed to be conserved.

The results were somewhat different for the two major OMPs. Among the 9 anti-OMP36 MAbs tested, only 5 reacted with rOMP36, and only 7 of the 13 anti-OMP25 MAbs reacted with rOMP25. Thus, about half of these MAbs appeared to be sensitive to conformational modifications and the antigenic structure appeared to be altered. However, some of the antibodies which remained reactive did react with intact *Brucella* cells. Therefore, it can be assumed that at least the epitopes specific for these MAbs can be recognized by polyclonal antibodies elicited during an infection, allowing the evaluation of the major OMPs as potential diagnostic tools.

Optimization of the ELISA protocol. Based on the results obtained with the MAbs, the recombinant antigens were diluted directly from the 6 M guanidine-HCl buffer into the coating buffer. For each antigen the optimal coating conditions were determined by using a pool of positive sera. Both the pH of the solution and the antigen concentration were optimized to obtain the highest sensitivity, the best possible signal-to-noise ratio, and the most economical use of the antigens (data not shown). To interpret the ELISA data, a cutoff value was calculated for each recombinant protein as the mean of the OD value plus three SDs, obtained with negative bovine sera (n = 96), negative ovine sera (n = 88), or negative caprine sera (n = 23) (Table 2). Except for the rp17 tested with the negative ovine sera, the cutoff values were rather low. In general, values for bovine and caprine sera were lower than those for ovine sera. The high cutoff value for the rp17 with ovine sera has yet to be explained, but a great heterogeneity in the reactivity of the sera was observed (SD = 0.203). When we used the calculated cutoff values, the specificity of the tests was generally well above 95%, with a slightly better score when negative ovine sera were studied. The animals which tested positive in these evaluations gave signals only slightly above the cutoff. Usually, it was not the same sample which was positive with different recombinant proteins. When sera from *Y. enterocolitica*-infected animals were tested on the recombinant proteins, none were reactive above the cutoff. These data show that the iELISA described above is sufficiently reliable, as far as specificity and sensitivity are concerned, to allow its use for evaluation of the recombinant proteins for serologic diagnosis.

Analysis of the antiprotein antibody response with sera from naturally infected animals. (i) Ovine sera. Ovine sera were assigned to four groups (A, B, C, and D) according to bacteriologic, serologic, and allergic data. In a preliminary step, sera belonging to each group (A, 10; B, 10; C, 5; and D, 5) were evaluated, in an iELISA, for their reactivity with each recombinant protein (Fig. 2). Whatever the antigen tested, an antiprotein response was detectable in some of the samples. In general, the higher the serologic response in the classic serologic tests, the higher the antiprotein response (i.e., the signals from group A and B samples were higher than those from groups C and D).

The weakest antibody response was that against OMP25, which detected only 33% of the animals tested. While detecting more of the positive animals, the OMP36 ELISA gave rather poor signals, with a signal-to-noise ratio rarely higher than 2. By contrast, the p15 and p39 antigens allowed detection of the majority of the animals (98%). In addition, while for the other antigens the positive responses often had a signal-to-noise ratio between 1 and 2, more than 50% of the positive samples scored higher than 2 when p15 or p39 was used.

For these reasons, these two antigens were further evaluated in combination, using sera from a larger number of animals in each group. The results are presented in Table 3. It can be seen

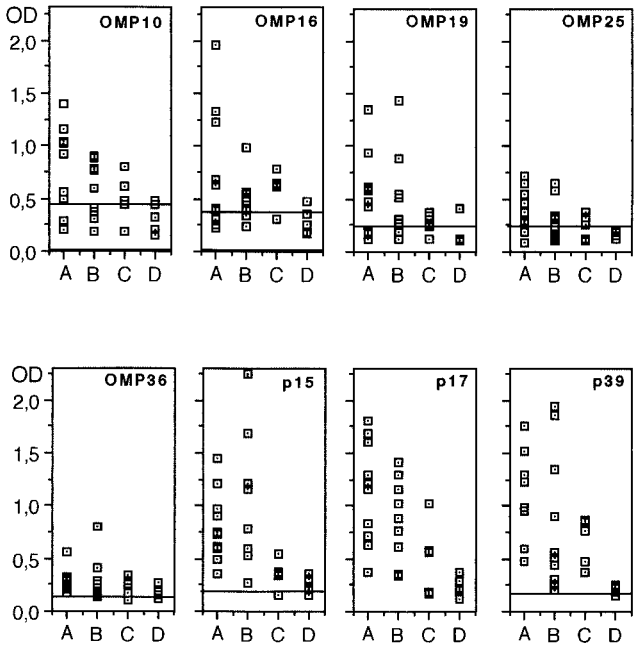

FIG. 2. Distribution of the iELISA OD values of eight recombinant *Brucella* proteins for 30 sheep from infected flocks. The horizontal axes refer to groups of animals (A, n = 10; B, n = 10; C, n = 5; D, n = 5) (see Materials and Methods). Each square corresponds to one animal. For each group, the animals analyzed were randomly selected. In each graph, the horizontal bar represents the calculated cutoff value for each ELISA.

TABLE 3. Comparison of results of iELISA, using recombinant *Brucella* proteins p39-p15 or p17, with other diagnostic tests on several groups of naturally infected sheep

Diagnostic test	% Positive animals in:				
	Group A[a] (n = 50)	Group B (n = 50)	Group C (n = 20)	Group D (n = 15)	Total (n = 135)
Bacteriology	100	0	0	0	37
Classic serology	100	100	0	0	74
DTH	100	100	100	0	88
S-LPS ELISA	100	98	95	80	96
p15-p39 ELISA	96	84	70	40	81
p17 ELISA	74	52	15	29	51

[a] Groups are defined according to bacteriologic, serologic, and DTH data (see Materials and Methods).

that most of the animals which are classified as negative on the basis of classic serologic tests (groups C and D) have significant anti-S-LPS antibody responses detected by the more sensitive S-LPS iELISA. Therefore, animals belonging to group D cannot be considered brucellosis-free.

The response against the mixture of recombinant products (p15-p39) appeared to be correlated with the strength of the anti-S-LPS response. Assuming that all sheep in the four groups (group D included) under study were infected, the sensitivity of the p15-p39 test is 81%, which is well above the sensitivity of the classic serologic tests, which identified only 74% of the positive samples. Only the DTH and the S-LPS ELISA performed better, with a sensitivity of 88 and 96%, respectively.

(ii) Goat sera. All animals (n = 54) tested were positive in classic serologic tests. The results obtained with the most relevant antigens are presented in Fig. 3. The p15 antigen alone or in combination with the p39 antigen allowed the identification of 95% of the positive animals tested. These results are in agreement with the results obtained with the ovine sera. The p17 antigen also performed quite well, since a sensitivity of 88% was obtained. However, all of the animals testing positive with the p17 antigen were also positive with the p15-p39 antigen combination, and therefore there was no complementarity between these two assays (data not shown).

(iii) Cattle sera. All samples (n = 47) were positive both in classic serologic tests and in the S-LPS ELISA. For the bovine species, the results were quite different from those found for the other ruminant species tested. As illustrated in Fig. 4, the best score was obtained with the p17 antigen, but less than 50% of the positive sera were identified. Moreover, the positive signals were rather weak in this test (data not shown). The p15 and p39 antigens detected less than 20% of the infected animals. By adding the results obtained separately with the p17 and the p39 antigens (calculated combination) a degree of complementarity was detected between these antigens which allowed identification of about 60% of the infected animals. The other antigens tested were not useful for detecting naturally infected cattle.

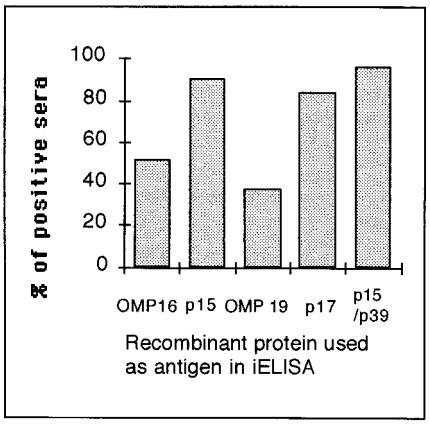

FIG. 3. Percentage of positive sera from naturally infected goats (n = 54) in iELISA on the best-performing recombinant *Brucella* antigens. All tested animals were positive in S-LPS serology.

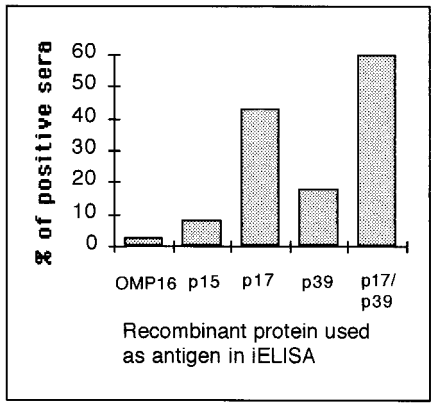

FIG. 4. Percentage of positive sera from naturally infected cattle (n = 36) in iELISA on the best-performing recombinant *Brucella* antigens. The p17-p39 results represent a calculated combination based on the results obtained on the individual antigens. All tested animals were positive in S-LPS serology.

Analysis of the antiprotein antibody response with sera from experimentally infected animals. To gain further insight into the diagnostic usefulness of the recombinant antigens, sera from experimentally infected animals were studied. This analysis allowed investigation of the effects of several parameters (e.g., infectious doses, pregnancy, etc.) on the antiprotein antibody response. In addition, only experimental infections allow a follow-up, over time, of the antiprotein antibody response and the time of its emergence relative to the development of anti-LPS antibodies.

(i) Ovine sera. Repeated sampling of 10 nonpregnant ewes from 1 week before infection to week 24 after infection allowed us to analyze the kinetics of the antibody response against the different recombinant antigens. Some prebleeding sera or sera collected up to 2 weeks after infection gave very low positive responses against some recombinant proteins (e.g., OMP16 and OMP10). Although seroconversion to S-LPS was readily detected by ELISA 2 and 4 weeks after infection, in 70 and 80% of the animals, respectively (data not shown), no significant antibody response was detected to any of the recombinant proteins tested even at the end of the experiment.

(ii) Goat sera. All animals tested were pregnant and became positive in S-LPS ELISA 23 to 26 days after infection. Figure 5 illustrates that, except for OMP25 and OMP36, ELISAs with the recombinant proteins demonstrated a clear increase in the number of positive animals with increased time postinfection. The best results were obtained with the OMP16, p15, and p17 antigens, confirming the data obtained with naturally infected goats. For these three antigens, approximately 80% of the animals tested positive at least once after the experimental infection (mostly after 1 or 2 months). Some animals were

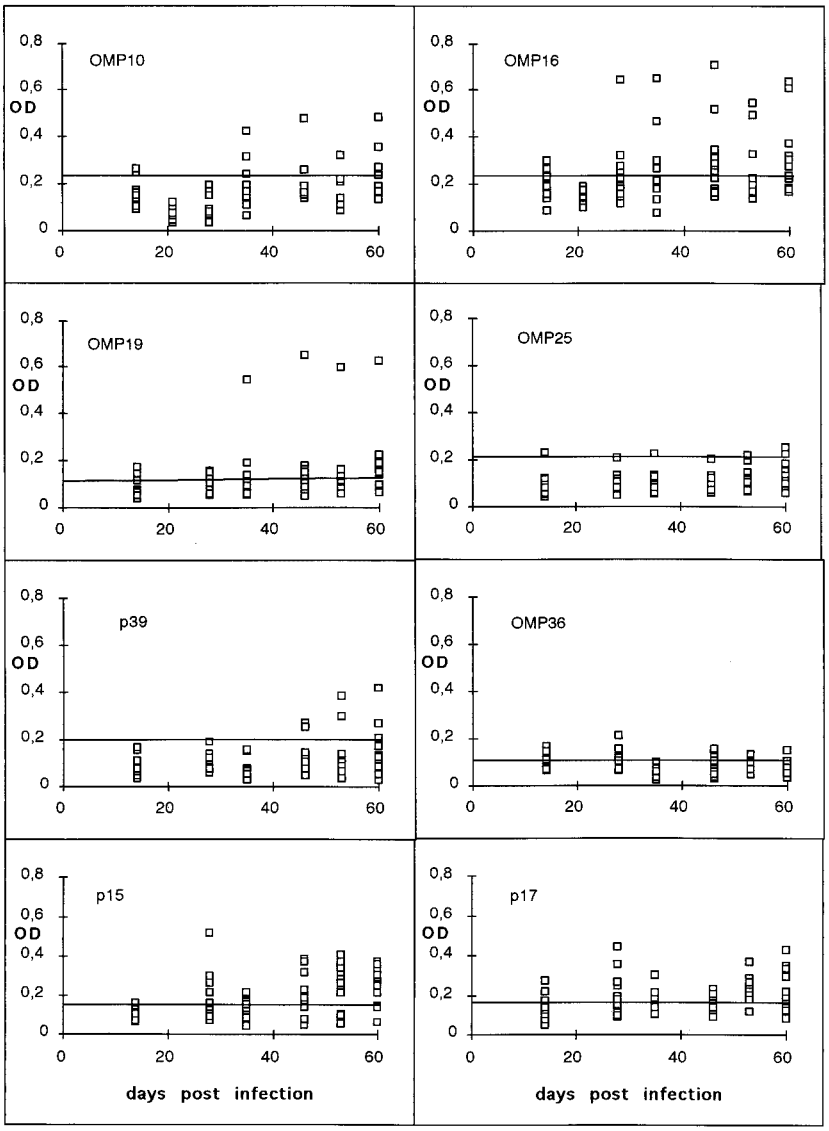

FIG. 5. Results obtained with sera from 12 experimentally infected goats at several times postinfection in iELISA for each *Brucella* recombinant antigen. Each square corresponds to one animal. In each graph, the horizontal bar represents the calculated cutoff value.

classified positive as early as 26 days postinfection with the OMP16 and p17 antigens.

(iii) Bovine sera. All sera collected weekly from the 24 pregnant heifers tested negative with most of the recombinant antigens (except p17) throughout the experiment (176 days). The p17 ELISA allowed the detection of 80% of the positive animals at days 99 and 132 postinfection, while only 50% remained positive at day 176 (Fig. 6). On these days all of the animals were clearly positive in the S-LPS ELISA (data not shown).

The positive results obtained with the p17 antigen were in agreement with the data obtained for the field infections, but no complementarity was observed with the p39 antigen. When these sera were tested in a competitive ELISA with a MAb directed against the p17, all the sera positive in the iELISA were also positive in the cELISA (23).

In another experiment, two groups of four nonpregnant heifers were infected with two different doses of *B. abortus* 544. All animals seroconverted as measured by S-LPS ELISA, but only one of the animals infected with the highest dose seroconverted to p17, albeit at a low titer. No reactivity against the other recombinant antigens was detected in these animals.

DISCUSSION

The live attenuated *Brucella* vaccines that are currently available induce a high-titer antibody response against the O polysaccharide of the LPS, which interferes with the serologic diagnosis of the disease. Therefore, the identification of protein components of *Brucella* bacteria which elicit an antibody response in the majority of infected animals, and which cross-react with all or most members of the genus, would improve diagnosis of the disease and contribute to the development of new vaccinal strategies.

Several *Brucella* proteins have been described as targets of the humoral immune response (2, 6, 19, 20, 23, 25, 37, 38). We

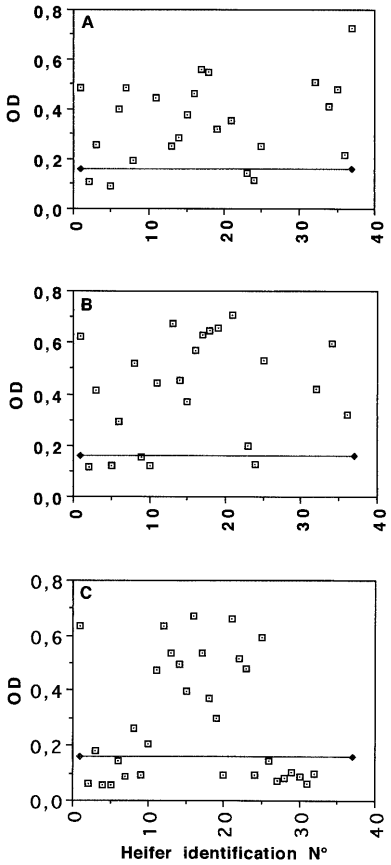

FIG. 6. iELISA on p17 recombinant antigen with sera from 24 experimentally infected heifers at the following times postinfection: (A) 99 days; (B) 132 days; (C) 176 days. The horizontal bar represents the calculated cutoff value.

selected eight of these proteins (five OMPs [10, 16, 19, 25, and 36] and three cytoplasmic proteins [p15, p17, and p39]) and evaluated their potential use for the serologic diagnosis of brucellosis. These proteins were expressed in *E. coli* as fusion polypeptides. The large-scale production and purification of these recombinant proteins provide large quantities of proteins devoid of all other contaminating *Brucella* macromolecules.

As a general conclusion, it is apparent that the antibody response against all OMPs was lower than the response against the cytoplasmic antigens regardless of the animal species or the type of infection (natural or experimental). This was particularly evident with the two major OMPs (OMP25 and OMP36). These results contradict previously published data which described these antigens as major antibody targets in various animal species (6, 14, 26).

Antigenic variations between the purified protein used as the antigen and the protein expressed in the infectious strains could, theoretically, explain such differences. However, except for *Brucella ovis* OMP25 (8), no antigenic variation has been described. In addition, where bacteriologic data are available, *B. ovis* was not isolated from the ovine or caprine brucellosis field cases studied here. Most probably, the discrepancies can be attributed to alteration of the antigenicity of the recombinant proteins, as demonstrated by the fact that about half of the MAbs tested did not recognize these recombinant products. In fact, these two proteins are complex integral membrane proteins whose antigenicity is dramatically influenced by quaternary structure (e.g., OMP36 is a trimeric protein) and/or their association with other membrane components (i.e., S-LPS). For example, two of the MAbs directed against the native OMP36 (A68/25G5/A5 and A63/15B6/C8) that failed to react with the recombinant OMP36 recognize the trimeric association of the porin (4) and cannot be expected to react with the recombinant product. In addition, for the two other antibodies (A63/5A7/A8 and A63/3H2/B1) that were negative with the recombinant OMP36, the epitopes were tentatively mapped on the N-terminal end of the protein, which is absent in the recombinant product (14).

Similarly, five of the seven MAbs which failed to recognize the recombinant OMP25 (A68/4B10/F5, A76/2C12/C11, A68/7D11/B3, A68/28G6/C7, and A70/6C5/B7) were described as highly reactive against intact *Brucella* cells in ELISA but only marginally reactive with denatured OMP25 in WB, suggesting that they are directed against discontinuous epitopes (8). Because serologic responses of naturally infected sheep against OMP25 and OMP36 allowed detection of 33 and 50%, respectively, of the infected animals, albeit at a low titer, these antigens may have some diagnostic value, provided their native conformation is conserved.

The antibody response against the minor OMPs (OMP10, OMP16, and OMP19) was somewhat greater. This is in agreement with the preservation of their antigenicity and is probably linked to the fact that they are not integral membrane proteins but lipoproteins (30, 31) and hence less likely to have their antigenicity influenced by the membranous environment. Among these, OMP16 performed best for detection of ovine and caprine field infections (about 50% of the animals having antibodies), followed by OMP10 and OMP19 for ovine and caprine serology, respectively.

The three cytoplasmic *Brucella* proteins tested (p15, p17, and p39) appeared to be the most promising antigens for the serologic diagnosis of brucellosis. This is in accordance with previous reports emphasizing the importance of the humoral immune response against cytoplasmic *Brucella* proteins (18, 19, 36). When we used sera from naturally infected animals, the best score was obtained with a combination of the p15 and p39 antigens, which allowed detection of 80 and 96%, respectively, of infected sheep and goats. The p17 antigen alone also performed rather well, because more than 50% of the sheep and more than 80% of the goats had antibodies against this protein. With regard to cattle, the p17 antigen appeared to be superior to any other antigen or antigen combination. For naturally infected sheep, the p15-p39 ELISA appeared to be a much more sensitive diagnostic test than bacteriology and the classic serologic tests. This ELISA, like the S-LPS ELISA, is complementary to the DTH test. Because the majority of the animals positive in the DTH test are also positive in ELISAs with the recombinant p15-p39 antigen, it could be argued that the serologic response may be a direct consequence of the skin test. In fact, the most commonly used allergen for skin testing is prepared from cytoplasmic extracts of *Brucella* sp. However, it seems that the effect of the DTH test on the serologic response can be ruled out for several reasons. First, even in group D, where no positive DTH reaction occurred, 40% of the animals were nevertheless positive in the ELISA. Second, in cattle or goats, which were also evaluated, no skin tests were performed and the cytoplasmic recombinant proteins remained the most reactive antigens. Finally, because we know that p39 is one of the constitutive proteins of Brucellergène, we performed seven successive DTH tests at 1-month intervals on several heifers, and the animals remained negative in the p39 ELISA (unpublished results). We have no similar data for the p15 and p17 antigens.

Compared to sheep and goats, cattle appear to produce

lower levels of antibodies against *Brucella* proteins. These differences could be linked to differences in host species as previously reported for *Brucella* sp. (31) and other pathogens (1). Lindler et al. (26) also reported that cattle do not produce any detectable antibody response against Omp28, while this antigen does elicit significant antibody responses in goats, rabbits, mice, and humans. Alternatively, such differences could be related to the prevalence of the disease in the region where the sera were collected. For instance, the sheep and goat sera were collected in areas where brucellosis is still endemic, and these sera gave a strong antiprotein antibody response. By contrast, the field bovine sera came from Belgium, where brucellosis has been almost eradicated, and these sera showed a low antiprotein response. This latter hypothesis is corroborated by the difference in reactivity of sera from naturally and experimentally infected animals.

Field-infected sheep gave a clear antiprotein response, and the intensity of the reaction was higher for animals in groups A and B, which were positive in all the diagnostic tests including (group A) or excluding (group B) bacteriology. By contrast, none of the nonpregnant ewes experimentally infected with *B. melitensis* showed a significant antibody response towards the recombinant antigens. Even ewes infected with a dose 10-fold higher did not develop a significant antibody response to these antigens (data not shown). Similar results were obtained with sera from experimentally infected nonpregnant cows. The difference in the immune response of naturally infected and experimentally infected animals may be due to differences in inoculation routes and doses of inocula, but more likely is caused by differences in disease pathogenesis and outcome. In fact, when pregnant goats were experimentally infected, an antiprotein response was demonstrated, sometimes as early as the anti-S-LPS response. This paralleled the observations made of field infections. To a lesser extent, the same was also true for experimentally infected pregnant cows, which showed a strong serologic response, but mostly towards the p17 recombinant protein. A good diagnostic reagent would most likely contain more than one immunoreactive protein to cover the spectrum of protein antibody response by ruminant species. Previous work (6, 23, 25, 29) demonstrated that a combination of immunodominant *Brucella* proteins would be able to detect most of the infected animals, whatever their species or genetic background. In this study, the best results were obtained by combining two recombinant cytoplasmic antigens: p15-p39 to test sera from ovine and caprine field cases and p17-39 for cattle field cases.

Other antigen combinations, including antigens not tested in this study (e.g., BP26 [28] and A2 [37]), should be evaluated in order to design a test with the best possible sensitivity. Nevertheless, the results reported here represent a first step towards the development of a multiprotein diagnostic reagent for brucellosis.

The specificity of the protein ELISA appeared to be excellent. However, the sensitivity of the protein ELISA is clearly insufficient when evaluated on sera from experimentally infected nonpregnant animals and could probably not be used in areas of low brucellosis prevalence, where the test should be able to certify the absence of the disease. Nevertheless, in endemic field situations, as illustrated by the naturally infected ovine and caprine cases, the ELISA with the p15 and p39 cytoplasmic antigens was able to detect more than 90% of the infected animals. Because of the high disease prevalence in these areas, vaccination is undertaken, and thus, interferences with diagnostic tests arise. In this context, an antiprotein ELISA that differentiates between titers due to vaccination and natural infection would be helpful.

Provided the diagnostic protein (or the protein combination) chosen is immunodominant after natural infection, the gene encoding this antigen could be deleted from a *Brucella* vaccine strain. Animals vaccinated with this engineered strain would not develop antibodies to the missing antigen. Therefore, this vaccination should be distinguished from natural infection by an ELISA with this antigen and should contribute to the eradication of brucellosis. Work is in progress to construct vaccine strains devoid of the relevant genes and to evaluate their vaccinal properties.

ACKNOWLEDGMENTS

We are very grateful to G. Dubray, A. Macmillan, B. Garin-Bastuji, C. Saegerman, and J. Blasco for providing sera. Cattle experimental infections were performed in the animal facilities of the National Institute for Veterinary Research (INRV) (Brussels, Belgium) under the supervision of J. Godfroid. We thank D. Gilson for her technical support and D. Prozzi for improving the English.

This research was supported by the Commission of the European Communities, contract ECLAIR AGRE-CT90-0049-C (EDB).

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