INFECTION AND IMMUNITY, Dec. 1994, p. 5376–5383
0019-9567/94/$04.00+0

Vol. 62, No. 12

A *Salmonella enteritidis* 11RX Pilin Induces Strong T-Lymphocyte Responses

ABIODUN D. OGUNNIYI,* PAUL A. MANNING, AND IEVA KOTLARSKI

Department of Microbiology and Immunology, The University of Adelaide, Adelaide, South Australia 5005, Australia

Received 20 May 1994/Returned for modification 4 July 1994/Accepted 10 September 1994

Our previous work, using proteins fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to define antigens of *Salmonella enteritidis* 11RX able to stimulate T cells from *S. enteritidis* 11RX-primed (BALB/c × C57BL/6)F_1 mice, had indicated the presence of a major antigenic determinant of 14 to 18 kDa (H.-M. Vordermeier and I. Kotlarski, Immunol. Cell. Biol. 68:299–305, 1990). The 14-kDa size is similar to that of the monomeric units of one of the fimbrial structures, SEF14, produced by a human enteropathogen, *S. enteritidis* 27655 (J. Feutrier, W. W. Kay, and T. J. Trust, J. Bacteriol. 168:221–227, 1986). Here we present data which indicate that *S. enteritidis* 11RX also produces this protein and that it is able to elicit delayed-type hypersensitivity reactions in *S. enteritidis* 11RX-primed animals and to stimulate in vitro proliferation of, and cytokine release from, T cells obtained from these animals, implying that this fimbrial protein is likely to be an important immunogen of *S. enteritidis*. The protein was purified to homogeneity and is free from contamination with lipopolysaccharide. Standard immunoblot analysis with unabsorbed *S. enteritidis* 11RX antiserum and antiserum absorbed with *Salmonella typhimurium* C5 and various strains of *Escherichia coli*, as well as a panel of anti-14-kDa-protein monoclonal antibodies, suggests that this fimbrial protein is not the common antigen expressed by a number of organisms belonging to the family *Enterobacteriaceae*. Immunogold electron microscopy with one of these monoclonal antibodies confirms that the 14-kDa protein and SEF14 are identical.

The increasing worldwide incidence of nontyphoid salmonellosis in humans caused by the invasive enteric pathogen *Salmonella enteritidis* (7) has attracted considerable attention in recent years (8, 12, 14, 29–32). Antigen-specific T-cell-mediated immunity is required for resistance to infection by this organism (19), as is the case with other intracellular bacterial parasites (IBPs) such as listeriae and mycobacteria, and effective protection depends on interaction between the $CD4^+$ ($L3T4^+$) and the $CD8^+$ ($Lyt2^+$) subsets of T cells (3, 16, 17).

We have demonstrated previously that T cells from *S. enteritidis* 11RX-immunized animals can be induced to proliferate in vitro and to release cytokines when cultured in the presence of antigen-presenting cells and formalin-killed 11RX organisms or soluble 11RX antigens (1, 19). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses have established that T-cell-stimulating antigens predominate in the region containing proteins of approximately 14 to 18 kDa but are also present in the 24-, 34-, and 50- to 60-kDa regions (39). The responding T cells are mainly of the $L3T4^+$ ($CD4^+$) phenotype, representing a mixed pool with a range of different antigenic specificities, reflecting the antigen preparations used to stimulate their responses.

The existence of two different pathways of antigen processing could explain why some fragmented peptides associate with class I major histocompatibility complex (MHC) products whereas others complex with class II molecules and why they elicit $CD8^+$ and $CD4^+$ T-cell responses, respectively (4, 11, 13, 25). It has been shown that when spleen cells are cultured with appropriate peptide fragments of ovalbumin (OVA), strong peptide-specific responses by $Lyt2^+$ ($CD8^+$) T cells are induced (5, 24). Fragments of OVA cleaved either by trypsin or by cyanogen bromide were effective, whereas native OVA did not provoke any detectable response (5). Interestingly, the introduction of OVA directly into the cytoplasm of EL4 tumor cells resulted in the presentation of this protein as OVA peptide-class I MHC molecule complexes (24). This approach could be exploited in the presentation of well-defined antigens of IBPs in the context of class I as well as class II MHC molecules of antigen-presenting cells (35, 38) and could enable a detailed evaluation of the relative importance of the two types of complexes in the induction of resistance to IBPs and an understanding of the biology of interaction of IBP antigens with antigen-presenting cells. To achieve this aim, it is essential to use purified antigens, preferably with relatively low molecular weights, thereby reducing the number of different antigenic determinants being studied.

The potential importance of fimbriae in pathogenesis and in the generation of host immunity to many species of the family *Enterobacteriaceae* is fast becoming a major focus of research (10, 18, 20, 28). As a continuation of our approaches to defining the antigens of 11RX which induce T-cell-mediated responses, we describe in this paper the purification and characterization of one such antigen, which is a fimbrial protein. We present data which establish that T cells of animals immunized with 11RX respond to a small fimbrial protein of 14 kDa which is unique to this organism. This protein is the mature form of the 16-kDa precursor polypeptide that we described previously as AP16 (40), and it corresponds to the 14-kDa structural subunit protein of *S. enteritidis* fimbriae (SEF14) recently characterized as *S. enteritidis* fimbrial subunit A (SefA) (6, 34).

MATERIALS AND METHODS

Animals. Sex-matched, 8- to 10-week-old BALB/c and (BALB/c × C57BL/6)F_1 (F_1) mice of both sexes were used for this study. Semi-lopeared rabbits provided the normal rabbit serum which was used as the source of complement (19). All

* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of Adelaide, Adelaide, S.A. 5005, Australia. Phone: (61 8) 3035434. Fax: (61 8) 3034362. Electronic mail address: dogunni@pene.science.adelaide.edu.au.

animals were obtained from the Central Animal House of the University of Adelaide.

Bacteria and bacterial antigens. *S. enteritidis* 11RX was originally obtained from D. Ushiba (36) and has been maintained in the laboratory since 1965 by using standard bacteriological procedures. It is a rough strain which is relatively avirulent for mice. The median lethal dose, determined after intravenous challenge of F_1 mice, is approximately 2×10^6 organisms (9). Exponential-phase cultures of 11RX were used for immunization of F_1 mice by intraperitoneal injection of 10^5 live organisms per mouse. The methods used to prepare the live organisms, formalin-fixed 11RX, and soluble extracts of 11RX have been described previously (19). *Escherichia coli* K-12 strains DH1, S17-1, and AAEC189 and *Vibrio cholerae* 569B were obtained from the departmental strain collection.

Fractionated nitrocellulose-bound antigens were prepared from soluble extracts of 11RX as described before (22). An antigenic preparation that was most stimulatory to T cells was located in the 14-kDa region of the soluble extract preparation and is herein referred to as AP14. The following concentrations of 11RX antigens were used for in vitro proliferation assays: formalin-fixed 11RX, 1 μg (dry weight) per ml; soluble extracts of 11RX, 10 μg of protein per ml; and AP14, 1 μg of protein per ml (as determined by the method of Lowry et al. [23]).

Media for lymphoid cell preparation and culture. Hanks' balanced salt solution supplemented with 100 IU of penicillin per ml and 100 μg of streptomycin per ml was used to harvest and prepare lymphoid cell suspensions. RPMI 1640 (Difco) with 2 mM glutamine, 0.1 mM 2-mercaptoethanol, 5 μg of indomethacin per ml, 10% (vol/vol) heat-inactivated fetal calf serum (Flow Laboratories), and antibiotics (see above) was used for all cell culture work and is referred to here as culture medium.

Medium for the cultivation of 11RX was CBT (Casamino Acids, vitamin B_1, tryptophan) agar freshly prepared by adding 50 ml of filter-sterilized minimal salts [K_2HPO_4, 70 g/liter; KH_2PO_4, 30 g/liter; $(NH_4)_2SO_4$, 10 g/liter (pH 7.5)], 12.5 ml of 20% Casamino Acids, 5 ml of 50% glucose, 5 ml of 1% $MgSO_4$, 5 ml of tryptophan, and 0.5 ml of 1-mg/ml vitamin B_1 to 450 ml of sterile molten water agar (7.5 g of BBL granulated agar, 450 ml of milliQ water). Aliquots (250 ml) of this agar were poured onto glass trays (290 mm^2), allowed to set and dry, and used immediately.

Preparation and purification of the 14-kDa protein. The large-scale method used to prepare 11RX protein was a modification of the methods described previously by Feutrier et al. (12) and Müller et al. (26) and consisted of subculturing 1-ml aliquots of an overnight CBT broth culture of 11RX, grown at 37°C with shaking, onto each of 10 glass trays (290 mm^2) containing 250 ml of CBT agar. Trays were incubated overnight at 37°C, and the bacteria on each tray were harvested with 25 ml of physiological saline to which 10 mM Mg^{2+} and 5 mM Ca^{2+} had been added. The 200 ml of bacterial suspension obtained was spun at $7,000 \times g$ for 5 min, and the pellet was washed once in the saline-Mg^{2+}-Ca^{2+} mixture. The pellet was thoroughly resuspended in the same solution, heated at 65°C for 30 min, cooled to room temperature, and centrifuged at $7,000 \times g$ for 20 min. The resulting supernatant was stored overnight at 4°C after 200 mg of sodium acetate and 0.001% sodium azide (NaN_3) were added. An aggregate, mainly lipopolysaccharide (LPS), was formed during this time; this aggregate and any free LPS were removed by ultracentrifugation at $200,000 \times g$ at 4°C for 90 min. The pellet was analyzed for protein and LPS contents to determine the amount of protein pelleted along with the LPS aggregate. To the supernatant (150 ml) was added ammonium sulfate [$(NH_4)_2SO_4$] to 50% saturation, and the solution was kept for 2 h at 4°C. The aggregate that formed (established to be the 14-kDa protein) was harvested by centrifugation at $20,000 \times g$ for 20 min at 4°C, resuspended in 10 ml of physiological saline, and dialyzed twice against 500 ml of saline in a dialysis tubing with a size cutoff of 10 kDa. The protein concentration of the solution was estimated by the optical density at 280 nm, using an Ultrospec Plus spectrophotometer (Pharmacia, LKB Biochrom, Milton Keynes, England) with bovine serum albumin (BSA) as the standard. The supernatant obtained from the last centrifugation was also saved for protein and LPS analyses to detect any residual protein or LPS.

Sample preparation and transfer for N-terminal sequencing. Large amounts of 11RX were prepared essentially as described above for the purification of the 14-kDa protein up to the heat elution step, with the exceptions that 11RX was grown on nutrient agar (Oxoid) and cells were washed and resuspended in phosphate-buffered saline (PBS) (pH 7.4). The heat-eluted protein was brought to room temperature and spun at $12,000 \times g$ for 10 min. The supernatant was precipitated with $(NH_4)_2SO_4$ to 50% saturation at 4°C for 2 h. The aggregate formed was collected by centrifugation at $12,000 \times g$ for 10 min and resuspended in PBS. This mixture was dialyzed for 2 h at 4°C against sterile distilled water to which 0.0002% NaN_3 had been added. A 2-ml aliquot of this protein was run in SDS on a 15% polyacrylamide gel and lightly stained before transfer to polyvinylidene difluoride (Bio-Rad) at 200 mA for 2 h in a Trans-Blot cell (Bio-Rad). The transfer buffer used was 25 mM Tris-HCl (pH 8.3)–192 mM glycine–10% (vol/vol) methanol. The protein bands transferred were stained for 10 min with Coomassie brilliant blue by standard procedures, and the membrane was sent to A. Gooley (Macquarie University, Sydney, Australia) for analysis on an Applied Biosystems 470A protein sequencer.

In vitro proliferation assays. Peritoneal lymphoid cell suspensions were prepared as previously described (19). For in vitro proliferation assays, cells were resuspended in culture medium and set up in quadruplicate in flat-bottom 96-well trays (Linbro 76 033 05) at a density per well of 1×10^5 nylon wool-fractionated peritoneal cells of immunized animals (IPC), with 2×10^4 normal peritoneal cells (NPC) as a source of antigen-presenting cells.

IL-2 assays. The interleukin-2 (IL-2)-dependent T-cell line cytotoxic lymphoid line (CTLL) was used to determine IL-2 levels in culture medium supernatants from 11RX-stimulated T cells. The supernatants assayed were harvested after 24 h of incubation of 1-ml cultures containing 1×10^6 IPC, 4×10^5 NPC, and the 11RX antigens being analyzed. The CTLL cells were set up at 5×10^3 per well with serial twofold dilutions of these culture medium supernatants in 0.2-ml volumes and incubated for 24 h. [^{3}H]thymidine (37 kBq per well) was added for the last 4 h of culture, and the amount of radioactivity incorporated was measured as described previously (19). Results are expressed as units per milliliter, calculated as described by Attridge and Kotlarski (1).

DTH assays and measurement of footpad swelling. Local transfer of delayed-type hypersensitivity (DTH) was measured by using freshly harvested nylon wool-fractionated IPC and cells from 3-day in vitro cultures of fractionated IPC. These were suspended to the required concentrations in culture medium without fetal calf serum and antibiotics. Cells (5×10^5 in 0.05 ml) were injected alone or with formalin-fixed 11RX (2.5 μg) or purified protein into the right hind footpads of recipient mice, and the thicknesses of both hind feet of the injected mice were measured up to 24 h by using dial gauge

calipers accurate to 0.05 mm (2, 3). In another experiment to demonstrate the antigenic activity of AP14 in vivo, groups of three 11RX-immunized mice were injected into one hind footpad with formalin-fixed 11RX (2.5 μg per mouse) or AP14 (1 μg per mouse). The footpad thicknesses of both hind feet were measured 24 and 48 h later and examined at 72 h.

Preparation and absorption of anti-11RX serum. Mice were immunized by intraperitoneal injection of 10^5 live 11RX organisms followed by three weekly intraperitoneal injections of 0.5 mg of formalin-killed 11RX starting 4 weeks later. The mice were bled a week after the last injection.

The serum obtained was extensively absorbed with *Salmonella typhimurium* C5 and *E. coli* K-12 strains DH1, S17-1, and AAEC 189 as follows. For each bacterial strain, cells were harvested and washed three times in physiological saline, and the pellet was resuspended at 5×10^{10} cells per ml in 2 ml of serum. Absorption was done seven times by alternating incubation at 37°C for 6 h in the presence of 0.0002% NaN_3 with incubation at 4°C overnight, using freshly harvested bacteria at each absorption step. After the last absorption, serum was dispensed into 0.4-ml aliquots and stored at −100°C.

Assay for effective absorption was done by colony and Western blotting (immunoblotting) with the nonabsorbed serum and normal mouse serum as primary antibody controls. The absorbed mouse anti-11RX was used as the primary antibody at a 1:500 dilution. The secondary antibody was sheep anti-mouse $F(ab)_2$ horseradish peroxidase (Amersham). The substrate used for detection was prepared by adding 15 mg of 4-chloro-1-naphthol (Sigma) to PBS with 20% methanol and 0.08% H_2O_2.

Preparation of anti-AP14 monoclonal antibodies. Five 8-week-old female BALB/c mice were primed for production of antibodies to the 14-kDa protein by injecting 100 μg of the purified protein in Freund's complete adjuvant at the base of the tail of each mouse. After 2 weeks, mice received a booster intravenous dose of the same antigen. They were given a final boost a week later and sacrificed 4 days after tertiary immunization.

Spleen cells and myeloma (X63) cells were prepared and fused according to standard methods (27). Plating was done in 96-well flat-bottom trays (Falcon 3072; Becton Dickinson), and medium was replenished at appropriate intervals. Apparent fusions were transferred to 24-well trays and maintained with fresh medium as required. Supernatants were screened for positive hybrids producing antibodies to the 14-kDa protein by enzyme-linked immunosorbent assay (ELISA), colony (dot) blotting, and reactions in Western blots. Each hybrid supernatant was concentrated 20-fold in an Amicon apparatus with a PM10 membrane. These served as primary antibodies in the immunoblotting assays. The secondary antibody was sheep anti-mouse $F(ab)_2$ horseradish peroxidase. The detection system was the same as that used for the polyclonal antiserum.

SDS-PAGE, colony (dot) blotting, and Western blotting. Samples from the protein purification step were analyzed by SDS-PAGE with 15% polyacrylamide gels and Coomassie brilliant blue staining (21). Immunostaining was performed after protein bands were transferred onto nitrocellulose filters.

For colony (dot) blotting, bacteria were grown to mid-exponential phase in CBT broth. They were washed once in physiological saline and then resuspended to 4×10^9 cells per ml in saline. About 10 μl of each bacterial suspension was spotted onto a nitrocellulose disc, allowed to air dry for 5 min, and then lysed with 0.5 M HCl for 30 min. Unlysed cells were removed with a jet stream of saline, and the disc was blocked with 5% (wt/vol) skim milk powder in 0.05% Tween 20 plus 20 mM Tris-buffered saline. Incubation with primary and secondary antibodies and detection were carried out as described above.

LPS (silver) staining. LPS (silver) staining was carried out to detect any LPS contamination of the purified protein. SDS-PAGE of samples was performed on a 15% polyacrylamide gel after digestion of protein with proteinase K (15). The gel was silver stained according to the method described by Tsai and Frasch (33).

Immunogold electron microscopy. 11RX was grown statically in CBT broth for 48 h at 37°C. The cells were resuspended in PBS to 10^{11} cells per ml, and 20 μl of each suspension was spotted onto a piece of Parafilm. Nickel grids (200 mesh; Graticules) coated with collodion were initially treated with 100 μg of poly-L-lysine per ml for 5 min and placed on each bacterial suspension for 5 min. The grids were subsequently blocked with 3% BSA in PBS for 3 min and then incubated with a 1:10 dilution of monoclonal antibody KAP14-1 in 3% BSA–PBS for 15 min. This was successively followed by two PBS washes, incubation with a 1:80 dilution of protein A-gold for 10 min, and two distilled water washes. The grids were finally negatively stained with 1% uranyl acetate for 30 s, blotted dry, and examined under a Philips CM 100 transmission electron microscope.

RESULTS

Purification of the 14-kDa protein. An extension of our various approaches to defining and characterizing the T-cell-stimulatory antigens of 11RX was the purification of its highly immunogenic 14-kDa fimbrial protein. This was accomplished by differential ultracentrifugation and ammonium sulfate precipitation as described in Materials and Methods. The 11RX organisms used to prepare this antigen were grown on CBT agar to increase fimbrial expression, thereby improving the yield of the protein. Furthermore, solid medium is a convenient way to handle and harvest bulk cultures, and it is more economical than broth cultures. The ultracentrifugation step was introduced to remove LPS from the protein preparation; however, SDS-PAGE analysis (Fig. 1A) shows that a substantial amount of the heat-eluted material, including some fimbrial protein, was also removed by this process. This loss was compensated for by the purity of the final preparation (the 14-kDa protein), which was confirmed by silver staining (Fig. 1B) to be devoid of LPS. The total protein yield from this procedure was estimated as 1.5 mg/g (dry weight) of bacteria, with BSA as the standard.

N-terminal amino acid sequence of AP14. Analysis of the amino acid sequence of AP14 established its homology to the published sequence FM$SALEN (Swiss Protein identification number) of the fimbrial protein of *S. enteritidis* (Table 1). The sequence blot gave 30 clear cycles with only two differences from the published sequence. These were the Glu/Val difference in position 9 and the Asn/Ser difference in position 27. The sequence data of Turcotte and Woodward (34) are similar to those reported here except for the Asn/Ser difference in position 27.

In vitro responses of normal and 11RX-primed T cells to AP14. LPS is mitogenic for B cells and is known to be a major contaminant of proteins which localize in the 14- to 18-kDa region of SDS-PAGE-fractionated preparations of salmonellae. It was important to ascertain that the biological activity of AP14 was not partly due to LPS contamination, because it is often difficult to prepare T-cell populations free of B cells. To rule out this possibility, a comparison of the in vitro abilities of purified AP14, formalin-fixed 11RX, soluble extracts of 11RX, and 11RX-LPS to stimulate 11RX-primed and normal T cells

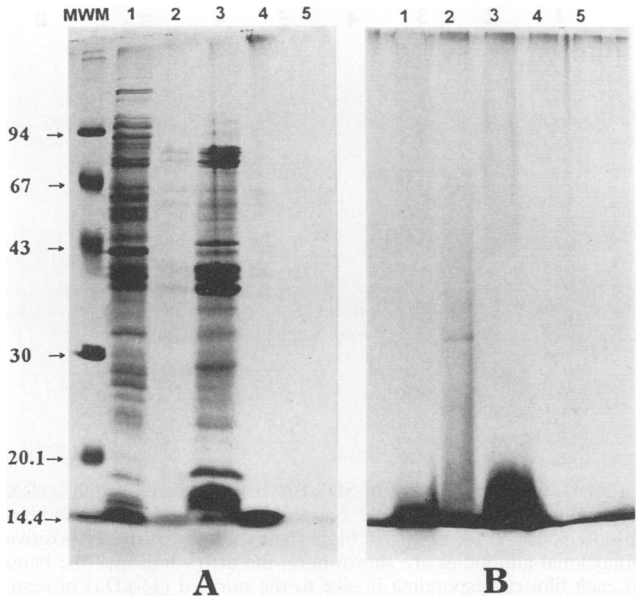

FIG. 1. (A) SDS-PAGE analysis of samples obtained during the purification of AP14. The proteins were visualized by staining with Coomassie blue. Lane MWM contains the low-molecular-weight markers (Pharmacia) phosphorylase *b*, BSA, OVA, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (from highest to lowest; molecular weights are shown in thousands). Other lanes: 1, whole 11RX preparation (1 mg of protein); 2, starting supernatant (heat-eluted fraction, 200 μg of protein); 3, LPS pellet (200,000 × *g*, 500 μg of protein); 4, AP14 (100 μg); 5, residual supernatant. (B) Same samples as in panel A but silver stained. Samples were treated with proteinase K before electrophoresis. Molecular weights are as in panel A.

was made. Control cultures containing normal or 11RX-primed T cells and the T-cell mitogen concanavalin A were included for comparison. AP14 induced 11RX-primed T cells to proliferate to a level similar to that observed with formalin-fixed 11RX or soluble extracts of 11RX, whereas 11RX-LPS had no stimulatory effect (Table 2); none of these antigens could stimulate purified T cells from normal animals (Table 3). As expected, both types of T cells were equally responsive to concanavalin A (Tables 2 and 3).

Purified AP14 (at 1 μg/ml) was also able to induce secretion of IL-2 into the supernatants of 11RX-primed T-cell cultures, to an extent comparable to that observed with soluble extracts of 11RX (at 10 μg/ml) (Table 4), corroborating the results obtained earlier with the partially purified protein (40). However, 11RX-LPS did not induce significant levels of IL-2 secretion (data not shown). These observations and the observation that no LPS was detected in the AP14 preparation by LPS staining of gels following SDS-PAGE fractionation (Fig. 1B) make it unlikely that LPS contributed to the antigenic activity of AP14. We observed some variability in the relatively high levels of IL-2 released from 11RX-primed T cells cultured with AP14 or soluble extracts of 11RX in the absence of NPC, ranging from almost undetectable levels to more than 50% of those released when NPC were also present. A likely explanation is that the 11RX-primed T cells were contaminated by small amounts of the antigen-presenting cells at variable levels. Since significant proliferation of all the T-cell preparations used in these repeat experiments was not obtained unless NPC were added, it follows that induction of cytokine release from these T cells requires fewer antigen-presenting cells than induction of proliferative responses.

TABLE 1. N-terminal amino acid sequences of the 14-kDa fimbrial proteins of *S. enteritidis* 11RX, 27655-3b, and 1246/89

Strain	Amino acid sequence[a]	Reference(s)
11RX	AGFVGNKAEVQAAVTIAAQNTTSANWNQDP	This paper
27655-3b	AGFVGNKAVVQAAVTIAAQNTTSANWSQDP	6, 12
1246/89	AGFVGNKAEVQAAVTIAAQNTTSANWSQDP	34

[a] For strain 11RX, the amino acid sequence was deduced chemically from the SDS-PAGE-purified protein by A. Gooley, Macquarie University, Sydney, Australia; for strains 27655-3b and 1246/89, the amino acid sequences were deduced by DNA sequencing. Underlining indicates amino acids that are not identical in all three sequences.

TABLE 2. In vitro proliferation of T cells from 11RX-immune mice

Stimulant[a]	[^{3}H]TdR incorporation (cpm, mean ± SEM) after 3 days of culture[b]		
	T cells	T cells + NPC	NPC
AP14 (1 μg/ml)	5,023 ± 496	43,522 ± 2,803	79 ± 22
F11RX (1 μg/ml)	5,798 ± 531	42,103 ± 3,301	101 ± 16
S11RX (10 μg/ml)	ND[c]	35,659 ± 980	89 ± 18
LPS (10 μg/ml)	697 ± 335	1,063 ± 489	153 ± 88
ConA (1 μg/ml)	23,217 ± 3,320	66,778 ± 3,885	106 ± 34
Medium only	66 ± 14	863 ± 232	52 ± 21

[a] F11RX, formalin-killed 11RX; S11RX, soluble extracts of 11RX; ConA, concanavalin A.
[b] 1×10^5 T cells and 2×10^4 NPC were used per well. Cultures were pulsed for 4 h with [^{3}H]thymidine ([^{3}H]TdR) prior to harvest. Data presented are for quadruplicate cultures.
[c] ND, not done.

DTH responses. Proliferative responses alone are insufficient to determine whether cell-mediated immunity to salmonellae has been mounted. We therefore employed DTH assays with AP14 as the eliciting antigen to demonstrate its antigenic activity in vivo. A local DTH reaction was elicited when AP14 added to fractionated IPC was injected into the hind footpads of nonimmunized mice, with maximum swelling occurring 24 h after injection (data not shown). Similarly, when injected into the left hind footpads of 11RX-immunized mice, AP14 elicited a significant DTH response, with maximum swelling occurring 48 h after injection (Table 4), little or no swelling occurring at 24 h, and swelling significantly reduced at 72 h (data not shown). The percent swelling was calculated by using the footpad thickness of the uninoculated (right) hind footpad of each test mouse as the baseline measurement. Comparable results were obtained with the partially purified protein (40).

TABLE 3. In vitro proliferation of T cells from nonimmune mice

Stimulant[a]	[^{3}H]TdR incorporation (cpm, mean ± SEM) after 3 days of culture[b]		
	T cells	T cells + NPC	NPC
AP14 (1 μg/ml)	1,563 ± 58	6,283 ± 593	79 ± 22
F11RX (1 μg/ml)	2,365 ± 389	10,120 ± 1,662	101 ± 16
S11RX (10 μg/ml)	ND[c]	3,222 ± 453	89 ± 18
LPS (10 μg/ml)	2,069 ± 653	5,344 ± 688	153 ± 88
ConA (1 μg/ml)	51,023 ± 2,998	60,038 ± 3,220	106 ± 34
Medium only	80 ± 16	ND	52 ± 21

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *b*.
[c] ND, not done.

TABLE 4. Ability of 11RX antigens to induce IL-2 production and elicit DTH reaction

Stimulant[a]	IL-2 released (U/ml)[b]			% Swelling at the following time after challenge[c]:	
	T cells	T cells + NPC	NPC	24 h	48 h
AP14 (1 μg/ml)	43	81	<2	23.5 ± 5.3	31 ± 7.7
S11RX (10 μg/ml)	35	67	<2	ND[d]	ND
F11RX (1 μg/ml)	ND	ND	ND	34.6 ± 2.2	55 ± 7.8
Medium only	<2	<2	<2	1.7 ± 1.0	0.6 ± 0.5

[a] See Table 2, footnote *a*.
[b] Samples tested were culture supernatants harvested after 24 h of incubation of 11RX-primed T cells, NPC, and antigen and assayed by using the CTLL cell line.
[c] Results are means ± standard errors of the mean for groups of three mice. The percent footpad swelling was calculated on the basis of measurements of right (control) and left (test) hind footpad thicknesses.
[d] ND, not done.

Preparation of anti-11RX serum. In an earlier paper (40), we observed that formalin-killed suspensions of other *Enterobacteriaceae*, such as *E. coli*, were able to induce proliferation of, and IL-2 release from, 11RX-primed T cells. This led to the suggestion that an 11RX antigen(s) localized in the 14- to 18-kDa region could be the common antigen(s) expressed by a number of *Enterobacteriaceae*. However, by extensive absorption of the antiserum raised against 11RX with C5 and several *E. coli* strains, we have demonstrated by dot blotting (Fig. 2A) that AP14 is unique to 11RX. The same results were obtained by Western blotting with this antiserum and whole-cell preparations of these bacteria, which also shows that the serum reacts specifically with AP14 of 11RX (Fig. 2B).

Monoclonal antibodies. A panel of monoclonal antibodies to AP14 was raised as described in Materials and Methods.

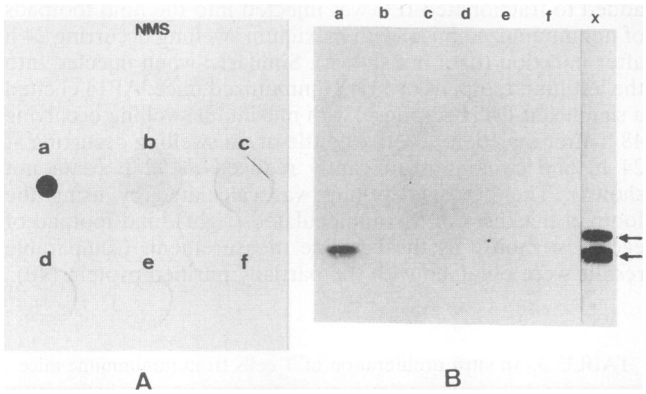

FIG. 2. (A) Dot blotting using an anti-11RX serum extensively absorbed with several species of *Enterobacteriaceae* against 11RX, other *Enterobacteriaceae*, and *V. cholerae*. NMS, normal mouse serum against 11RX as a negative control; a, 11RX; b, *S. typhimurium* C5; c, d, and e, *E. coli* K-12 strains DH1, S17-1, and AAEC189, respectively; f, *V. cholerae* 569B. The serum is specific for 11RX. (B) In a Western blot of whole-cell preparations of these bacteria with this antiserum, this specificity corresponds to a band in the whole 11RX preparation with the size of the purified protein. x, whole-cell preparation of *E. coli* K-12 strain AAEC189 carrying an 11RX cosmid; other letters are as in panel A. Both the precursor (16-kDa, top arrow) and the mature (14-kDa, bottom arrow) forms of SEF14 protein react with monoclonal antibody KAP14-1.

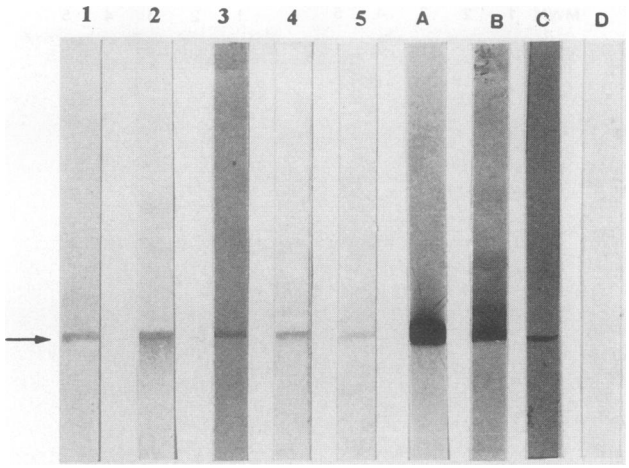

FIG. 3. Western blotting of SDS-PAGE-fractionated whole 11RX preparations, showing specific reactivity of monoclonal antibodies raised against AP14. Only five blots (lanes 1 to 5) of the 11 positive monoclonal antibodies are shown here; the arrow indicates the band on each blot corresponding in size to the purified (14-kDa) protein. Lane A, purified AP14 blotted with one of the monoclonal antibodies; lane B, whole 11RX preparation blotted with anti-11RX polyclonal serum for comparison; lane C, purified AP14 stained with 1% amido black; lane D, hybrid supernatant negative for antibody to the 14-kDa protein.

The supernatants that were positive by ELISA were further screened by colony and Western blotting. Of the supernatants tested, 11 reacted positively to the whole 11RX preparation by the dot blot method (data not shown). The specific reactivity of these antibodies to AP14 was further tested by Western blotting of an SDS-PAGE-fractionated whole 11RX preparation and the purified protein under reducing conditions. Figure 3 shows, using five representative blots, that the monoclonal antibodies bound specifically to one band of the whole 11RX preparation; this band corresponded to the size of the purified protein. The monoclonal antibodies also reacted, but to a lesser extent, to a band at the top of the polyacrylamide gel. Presumably, this represents the residual protein that did not run through the stacking gel completely.

Immunogold electron microscopy. 11RX organisms were subjected to immunoelectron microscopy with one of the monoclonal antibodies raised against the purified 14-kDa fimbrial antigen described here. Figure 4 shows that the antibody binds specifically to the fimbrial antigen, further confirming that the 14-kDa protein corresponds to the SefA fimbrial protein of SEF14 described by Clouthier et al. (6).

DISCUSSION

A requirement for an effective evaluation of the relative importance of class I and class II MHC molecules in the presentation of well-defined *Salmonella* antigens for induction of T-cell-mediated immunity is the availability of purified antigens, preferably with relatively low molecular weights. They are needed not only to reduce the number of different antigenic determinants but also to abolish nonspecific proliferation of lymphoid cells from immune mice due to contaminating LPS in protein antigen preparations (37). In this paper, we describe the purification and immunogenic characteristics of one such antigen, a 14-kDa fimbrial protein of *S. enteritidis* 11RX.

Purification of the 14-kDa protein was achieved by differen-

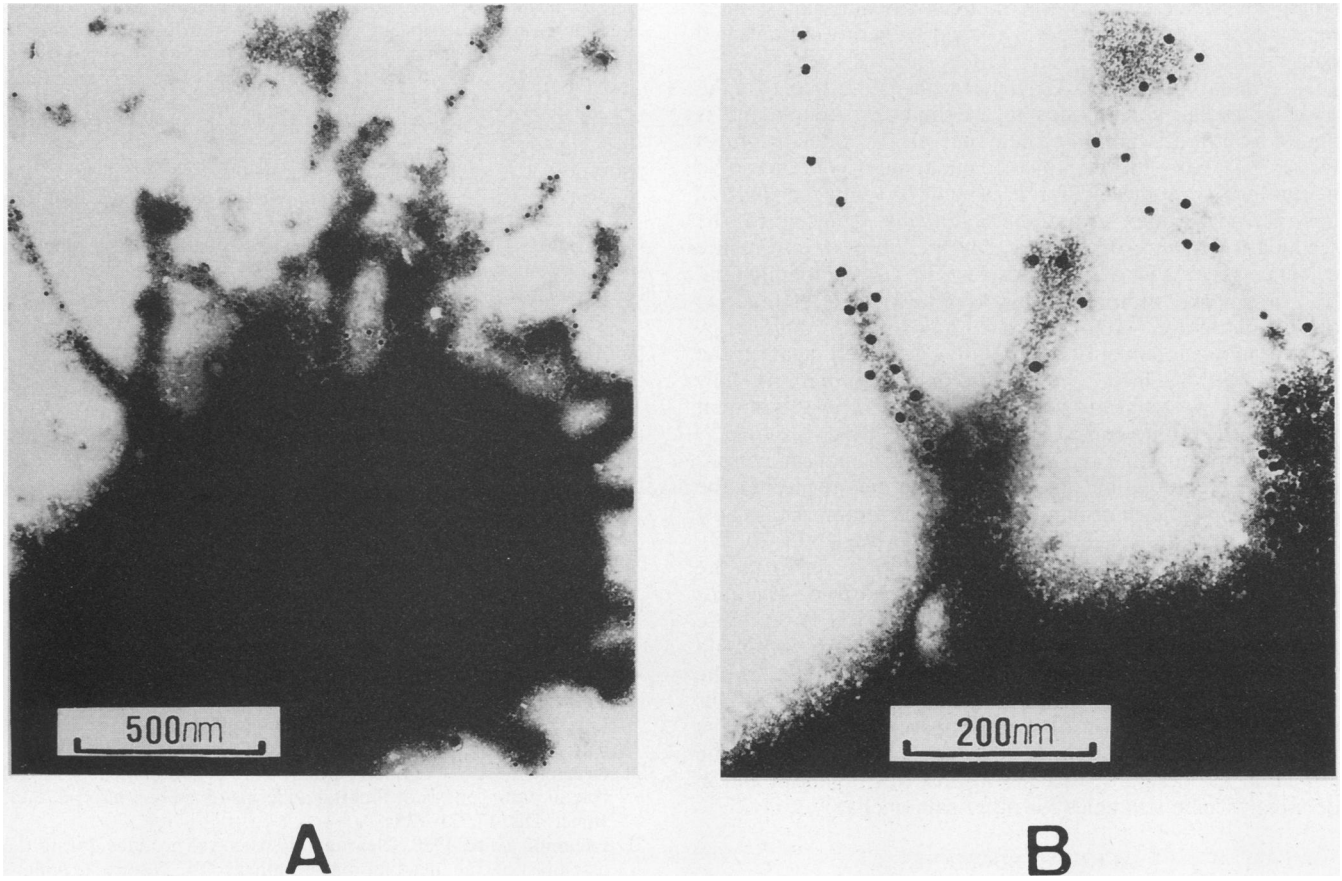

FIG. 4. Immunoelectron microscopy of uranyl acetate-stained 11RX cells labelled with protein A-gold after incubation with monoclonal antibody KAP14-1, which recognizes AP14. Cells were grown in static CBT broth for 48 h at 37°C. Magnifications, ×28,500 (A) and ×73,000 (B).

tial ultracentrifugation and ammonium sulfate precipitation. The method we adopted for purification was developed by modification of a protocol published by others (12, 26) and was aimed at obtaining reasonable yields of the protein while reducing LPS contamination. The results we obtained indicate that the purification procedure was successful and reproducible, and the final product is satisfactory with regard to its homogeneity and suitability for immunological assays. In our previous attempts, we had used nutrient agar to grow the 11RX used to prepare this protein. However, when N-terminal amino acid analysis indicated the protein to be a fimbrin, we changed to using CBT agar because 11RX organisms grown on this medium produce more of the same fimbrial protein when grown under similar conditions. We ascertained this by comparing yields from both preparations; SDS-PAGE and Western blotting of both preparations with monoclonal antibody KAP14-1 indicated that both preparations contained the same fimbrial protein. Moreover, the N-terminal amino acid sequences of the corresponding bands are also the same. As a further step toward obtaining higher yields of the protein, molecular techniques are currently being used to overproduce the fimbrial protein and affinity purify the product by using the monoclonal antibodies described here.

The protein appears to be an important immunogen of 11RX because the in vitro proliferative response and levels of IL-2 released from 11RX-primed T cells in the presence of purified AP14 are comparable to those for formalin-fixed 11RX and soluble extracts of 11RX. Levels of proliferation of T-cell lines and clones to the partially purified form of the protein and formalin-fixed 11RX and soluble extracts of 11RX have been previously found to be very similar. These observations confirm our earlier evidence with the T-cell Western blot technique (40) employed in selecting specific AP14-reactive T-cell clones, using mice immunized against whole 11RX and maintained with the partially purified protein, that AP14 is highly immunogenic. In addition, the demonstration that AP14 was able to elicit a DTH reaction in vivo confirms our suggestion that this protein could be an important immunogenic determinant of 11RX (39, 40). Standard immunoblot analysis with an *S. enteritidis* 11RX antiserum extensively absorbed with C5 and several *E. coli* strains, as well as with a panel of anti-AP14 monoclonal antibodies, suggests that this protein is not a common antigen expressed by a number of organisms belonging to the *Enterobacteriaceae* as we implied in our earlier paper (40). However, these data are not absolutely conclusive because antibodies are not necessarily able to characterize cross-reacting T-cell epitopes. Nevertheless, these antibodies will be useful in preliminary and comparative molecular and immunological assays aimed at characterizing this protein further and in defining its T-cell epitopes.

Our previous publications (39, 40) had identified this protein to be 16 kDa in size, and we thus referred to it as AP16. Further molecular work with this protein by using *E. coli* recombinant clones and Western blot analysis with the monoclonal antibodies to the 14-kDa form has revealed that the 16-kDa form is the precursor form of the protein, since both

bands are detected with the monoclonal antibodies. Such reactions were not observed in control bacteria (unpublished data).

N-terminal amino acid sequencing has confirmed that the 14-kDa protein corresponds to the fimbrial subunit and is homologous to the SEF14 protein recently described by others (6, 12, 34), except for the indicated amino acid differences. We intend to confirm these differences by DNA sequence analysis; however, they do not seem to be potentially significant for the secondary structure or the reactivity of the protein. Further evidence that AP14 corresponds to SEF14 is the demonstration of fimbriae morphologically resembling SEF14 fimbriae on the surface of 11RX cells by immunoelectron microscopy with a monoclonal antibody, KAP14-1, raised against the purified AP14. Once the identity of this protein is fully established, we intend to refer to it as SEF14 in subsequent work in order to be consistent with the other publications.

It is interesting that the 11RX protein is a fimbrin, considering the accumulating data being generated regarding the potential importance of immune responses to fimbriae in host immunity to many species of *Enterobacteriaceae* (9, 18, 20, 28). In addition, there has been a worldwide increase in the incidence of nontyphoid salmonellosis caused by *S. enteritidis* in humans in recent years (8, 12, 14, 29–32). It is therefore logical to speculate that further work with this protein should allow a more detailed assessment and understanding of the role of this protein in the biology of *S. enteritidis* and its significance in induction of resistance to this organism. The results from the T-cell epitope mapping studies would also be critical in the design of vaccines against this organism and in devising vaccine strategies for other salmonellae.

ACKNOWLEDGMENTS

We thank G. Penney for excellent technical assistance, T. Proctor for photography, J. Iredell for help with electron microscopy, and A. Gooley for N-terminal sequencing of the 14-kDa protein.

This work was supported by a research grant from the National Health and Medical Research Council of Australia. A. D. Ogunniyi is supported by the Equity and Merit Scholarship Scheme administered by the Australian International Development Assistance Bureau.

REFERENCES

1. **Attridge, S. R., and I. Kotlarski.** 1985. In vitro lymphokine release by lymphocytes from mice infected with *Salmonella*. Aust. J. Exp. Biol. Med. Sci. **63**:489–502.
2. **Attridge, S. R., and I. Kotlarski.** 1985. Local transfer of delayed-type hypersensitivity after *Salmonella* infection in mice. Infect. Immun. **50**:807–812.
3. **Boom, W. H., R. N. Husson, R. A. Young, J. R. David, and W. F. Piessens.** 1987. In vivo and in vitro characterization of murine T cell clones reactive to *Mycobacterium tuberculosis*. Infect. Immun. **55**:2223–2229.
4. **Braciale, T. J., L. A. Morrison, M. T. Sweester, T. Sambrook, M. J. Gething, and V. L. Braciale.** 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. Immunol. Rev. **98**:95–114.
5. **Carbone, F. R., M. W. Moore, J. M. Sheil, and M. J. Bevan.** 1988. Induction of cytolytic T lymphocytes by primary in vitro stimulation with peptides. J. Exp. Med. **167**:1767–1779.
6. **Clouthier, S. C., K.-H. Müller, J. L. Doran, S. K. Collinson, and W. W. Kay.** 1993. Characterization of three fimbrial genes, *sefABC*, of *Salmonella enteritidis*. J. Bacteriol. **175**:2523–2533.
7. **Cohen, M. L., and E. J. Gangarosa.** 1978. Nontyphoid salmonellosis. South. Med. J. **71**:1540–1545.
8. **Cooke, E. M.** 1990. Epidemiology of foodborne illness: UK. Lancet **336**:790–793.
9. **Davies, R.** 1975. A study of the carrier state in *Salmonella* infection. Ph.D thesis. University of Adelaide, Adelaide, Australia.
10. **Duguid, J. P., and D. C. Old.** 1980. Adhesive properties of *Enterobacteriaceae*, p. 185–227. *In* E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall Ltd., London.
11. **Falo, L. D., K. Sullivan, B. Benacerraf, M. Mescher, and K. L. Rock.** 1985. Analysis of antigen presentation by metabolically inactive accessory cells and their isolated membranes. Proc. Natl. Acad. Sci. USA **82**:6637–6651.
12. **Feutrier, J., W. W. Kay, and T. J. Trust.** 1986. Purification and characterization of fimbriae from *Salmonella enteritidis*. J. Bacteriol. **168**:221–227.
13. **Germain, R. N.** 1986. The ins and outs of antigen processing and presentation. Nature (London) **322**:687–689.
14. **Goodnough, M. C., and E. A. Johnson.** 1991. Control of *Salmonella enteritidis* infections in poultry by polymyxin B and trimethoprim. Appl. Environ. Microbiol. **57**:785–788.
15. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogenicity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
16. **Kaufmann, S. H. E.** 1988. $CD8^+$ T lymphocytes in intracellular microbial infection. Immunol. Today **9**:168–174.
17. **Kaufmann, S. H. E., E. Hug, U. Väth, and I. Müller.** 1985. Effective protection against *Listeria monocytogenes* and delayed-type hypersensitivity to listerial antigens depend on cooperation between specific $L3T4^+$ and $Lyt2^+$ T cells. Infect. Immun. **48**:263–266.
18. **Korhonen, T. K., R. Virkola, B. Westurlund, H. Holthöfer, and F. Parkkinen.** 1990. Tissue tropism of *Escherichia coli* adhesins in human extraintestinal infections. Curr. Top. Immunol. Microbiol. **151**:115–127.
19. **Kotlarski, I., M. Pope, K. Doherty, and S. R. Attridge.** 1989. The in vitro proliferative response of lymphoid cells of mice infected with *Salmonella enteritidis* 11RX. Immunol. Cell Biol. **67**:19–29.
20. **Krogfelt, K. A.** 1991. Bacterial adhesion: genetics, biogenesis and role in pathogenesis of fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **13**:721–735.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–683.
22. **Lee, S. P., W. G. Stokes, K. A. Grant, et al.** 1989. Cellular immune response of leprosy contacts to fractionated *Mycobacterium leprae* antigens. Infect. Immun. **57**:2475–2480.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
24. **Moore, M. W., F. R. Carbone, and M. J. Bevan.** 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell **54**:777–785.
25. **Morrison, L. A., A. F. Lukacher, V. L. Braciale, D. Fan, and T. J. Braciale.** 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J. Exp. Med. **163**:903–921.
26. **Müller, K.-H., S. K. Collinson, T. J. Trust, and W. W. Kay.** 1991. Type 1 fimbriae of *Salmonella enteritidis*. J. Bacteriol. **173**:4765–4772.
27. **Oi, V. T., and L. A. Herzenberg.** 1980. Hybridoma production, p. 351–355. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman, San Francisco.
28. **Pearce, W. A., and T. M. Buchanan.** 1980. Structure and cell membrane-binding properties of bacterial fimbriae, p. 289–344. *In* E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, series B, vol. 6. Chapman and Hall Ltd., London.
29. **Pohl, P., P. Lintermans, M. Marin, and M. Coutrier.** 1991. Epidemiological study of *Salmonella enteritidis* strains of animal origin in Belgium. Epidemiol. Infect. **106**:11–16.
30. **Rivera, M. J., N. Rivera, J. Castillo, M. C. Rubio, and R. Gómez-Lus.** 1991. Molecular and epidemiological study of *Salmonella* clinical isolates. J. Clin. Microbiol. **29**:927–932.
31. **Rodrigue, D. C., R. V. Tauxe, and B. Rowe.** 1990. International increase in *Salmonella enteritidis*: a new pandemic? Epidemiol. Infect. **105**:21–27.
32. **Todd, E.** 1990. Epidemiology of foodborne illness: North America. Lancet **336**:788–790.
33. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for

detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.

34. **Turcotte, C., and M. J. Woodward.** 1993. Cloning, DNA nucleotide sequence and distribution of the gene encoding the SEF14 fimbrial antigen of *Salmonella enteritidis*. J. Gen. Microbiol. **139:**1477–1485.
35. **Unanue, E. R.** 1984. Antigen-presenting function of the macrophage. Annu. Rev. Immunol. **2:**395–428.
36. **Ushiba, D., K. Saito, T. Akiyama, M. Nakano, T. Sugiyama, and S. Shirono.** 1959. Studies on experimental typhoid: bacterial multiplication and host cell response after infection with *Salmonella enteritidis* in mice immunised with live and killed vaccines. Jpn. J. Microbiol. **3:**231–242.
37. **Villarreal, B., P. Mastroeni, R. D. de Hormaeche, and C. E. Hormaeche.** 1992. Proliferative and T-cell specific interleukin (IL-2/IL-4) production responses in spleen cells from mice vaccinated with *aroA* live attenuated *Salmonella* vaccines. Microb. Pathog. **13:**305–315.
38. **von Boehmer, H., P. Kisielow, H. Kishi, B. Scott, P. Borgulya, and H. S. Teh.** 1989. The expression of CD4 and CD8 accessory molecules on mature T cells is not random but correlates with the specificity of the β receptor for antigen. Immunol. Rev. **109:**143–151.
39. **Vordermeier, H.-M., and I. Kotlarski.** 1990. Identification of antigens which stimulate T lymphocytes of *Salmonella enteritidis* 11RX immunized mice. Immunol. Cell. Biol. **68:**299–305.
40. **Vordemeier, H.-M., and I. Kotlarski.** 1990. Partial purification and characterisation of low molecular weight antigens of *Salmonella enteritidis* 11RX. Immunol. Cell Biol. **68:**307–316.