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Isolation and Characterization of Lymphocytes from Bovine Intestinal Epithelium and Lamina Propria

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ABSTRACT

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The lymphocyte populations of the bovine gut lamina proprial (LP) and epithelial tissues were isolated and characterized with respect to cells bearing surface and cytoplasmic immunoglobulin (Ig). Functional characteristics of cells from the two tissues, including responsiveness to Concanavalin A (Con A), anti-bovine immunoglobulin (anti-Ig), Con A supernatants of bovine peripheral blood lymphocytes (bConA sup) and recombinant human IL-2 (rhIL-2), were also assessed.

Less than 1% of the mononuclear cells in the epithelial tissue (IEL) stained for cytoplasmic Ig, and 9% stained positively for surface Ig. IEL did not proliferate in response to anti-Ig, although cells of this population did respond to Con A, bConA sup, and rhIL-2. Twenty-seven percent of bovine gut LP lymphocytes stained for surface Ig, while 39% of these cells were positive for cytoplasmic Ig. LP lymphocytes proliferated in response to all four stimulants used, Con A, anti-Ig, bConA sup and rhIL-2.

INTRODUCTION

During the last several years, interest in the development of novel approaches to livestock disease therapy and prophylaxis has been increasing. This development requires better understanding of populations of immunocompetent cells, their activities and their soluble products. Considerable progress has been made in the understanding of bovine systemic responses, including phenotypes of T cell subsets (Grewal et al., 1978; Baldwin et al., 1986; Splitter and Eskra, 1986), alloreactivity of peripheral blood mononuclear cells (Teale et al., 1986), and their IL-2 responsiveness (Miller-Edge and Splitter, 1984; Carter et al., 1986; Fong and Doyle, 1986; Picha and Baker, 1986). However, little is known about populations of lymphocytes isolated from the bovine gut. By analogy with murine systems (Nencioni et al., 1983; Carman et al., 1986),
the mucosal immune response can be expected to play an important role in defense against a variety of bacterial, helmintic and viral pathogens of cattle which invade or colonize mucosal surfaces.

Relatively pure populations of intraepithelial leukocytes (IEL) and lamina proprial (LP) lymphocytes have been obtained from the epithelium and lamina propria of the rodent and porcine small intestines using the methods of Davies and Parrott (1981a) and Leventon et al. (1983). Cells from these two compartments have been characterized with respect to morphology (Davies and Parrott, 1981a; Ernst et al., 1985), cell surface phenotype (Petit et al., 1985; Ernst et al., 1986), and functional activities (Davies and Parrott, 1980; Tagliabue et al., 1981; Mowat et al., 1983, 1986; Petit et al., 1985; Wilson et al., 1986b; Carman et al., 1986). These studies in the mouse have demonstrated that, not only do IEL and LP lymphocyte populations differ qualitatively in terms of resident immune effector cells, but quantitative differences in cytotoxic activity and responses to mitogens also exist.

The aim of the present studies was to develop an in vitro model for the study of mucosal immune responses in a ruminant species, the bovine. Techniques similar to those used to obtain cells from the murine gut were adapted to the isolation of lymphocytes from bovine gut epithelium and lamina propria. B lymphocytes from both compartments were identified by the presence of surface immunoglobulin. Cytoplasmic immunoglobulin was used as a marker for antibody-producing plasma cells. Mitogenic responses and proliferation upon stimulation with the T cell mitogen, Con A, anti-bovine immunoglobulin, bovine Con A supernatants, and recombinant human IL-2 were assessed. The results show that, as in rodent species, differences are apparent in bovine lymphocyte populations from the two compartments. The Con A mitogenic response of bovine gut epithelial lymphocytes is similar to that observed for porcine IEL and contrasts to that reported for murine IEL. Finally, bovine LP and IEL, like bovine peripheral blood lymphocytes, are very sensitive to rhIL-2 stimulation, suggesting that this lymphokine is involved in the regulation of the mucosal immune response in this species.

MATERIALS AND METHODS

Media

All phases of the IEL and LP separations were done in calcium- and magnesium-free (CMF) Hanks, (Gibco), supplemented with 10 μg/ml gentamicin (Sigma), 50 units/ml penicillin (Gibco) and 50 units/ml streptomycin (Gibco). Cells were cultured in RPMI 1640 (Gibco), supplemented with 10% fetal calf serum (FCS) (Hyclone), 10 μg/ml gentamycin, 50 units/ml penicillin, 50 units/ml streptomycin, 10 mM HEPES, 5 × 10⁻⁵ M 2-mercaptoethanol, and 2 mM L-glutamine (Gibco). Discontinuous gradients of isotonic Percoll
(Pharmacia), pH 7.2, were made as follows: 5 ml 80%, 7.5 ml 70%, 7.5 ml 55%, 15 ml 40% and 10 ml 30% Percoll.

Reagents

Concanavalin A (Type IV, Sigma) was diluted to 1 mg/ml in saline and stored at 4°C. Recombinant human IL-2 (Biogen) was stored at −70°C at 1 μg/ml in 25 mM acetic acid−0.5% BSA. Con A supernatants were a gift from Carl Edwards (Pitman-Moore, Inc.). They were made by incubating bovine peripheral blood monocytes with 5 μg/ml Con A for 72 h and inactivating the supernatants with methylalpha-D-mannopyranoside. The supernatants were shown to contain bovine macrophage-activating factor and bovine IL-2 (C. Edwards, personal communication, 1987). Anti-bovine immunoglobulin was prepared by immunizing rabbits with the immunoglobulin fraction of normal bovine serum. Rabbit serum was precipitated two times with a 40% saturated ammonium sulfate solution. The precipitate was dissolved, dialyzed into PBS, clarified by centrifugation, and stored frozen as a 5 mg/ml solution. Rabbit anti-bovine Ig was affinity purified for conjugation with fluorescein isothiocyanate (FITC). Fifteen micrograms FITC per milligram Ig were conjugated at pH 9.5, at 5°C for 18 h. The conjugate was dialyzed against 0.01 M phosphate to remove unbound FITC, and fractionated on a DEAE sepharose column. A conjugate with a fluorescein:Ig ratio of 5:1 was used in staining.

Cell separation

Both IEL and LP lymphocytes were obtained from small intestines of slaughterhouse steers by a modification of the method of Davies and Parrot (1981a). To isolate IEL, small intestines were cut open longitudinally, and rinsed under running water. Intestines were dipped in 95% ethanol, rinsed in CMF Hanks and cut into 1-inch-square (2.5 cm²) pieces. The pieces were washed in CMF Hanks and transferred to siliconized flasks containing 50 ml CMF Hanks-2% FCS. The flasks were incubated for 30 min at 37°C in a shaking water bath, and shaken vigorously to suspend the epithelial layer. The supernatants were removed, pooled, filtered over glass wool columns and washed. The incubation was repeated two times. Four × 10⁷ cells/ml were resuspended in 30% Percoll and layered on each gradient. The gradients were spun for 20 min at 37°C and 600g. Cells from the interfaces were collected and washed. The percent cell viability, determined by trypan blue exclusion, was 65–70%.

To obtain lamina proprial lymphocytes, pieces of intestine were incubated with stirring at room temperature in CMF Hanks−5 mM EDTA (Sigma) to
remove the epithelium. The supernatant was removed every 15 min, and media replaced until the supernatant was free of cells, about 90 min. The pieces were washed with CMF Hanks–2% FCS, incubated for 20 min in CMF Hanks–5% FCS to inactivate the remaining EDTA, and transferred to flasks containing 150 ml CMF Hanks–5% FCS with 10 units/ml Worthington CLS III Collagenase and an equal weight of soybean trypsin inhibitor (Sigma). The flasks were incubated for 75 min in a 37 °C shaking water bath, and 15 min on a magnetic stirrer. The supernatants were removed, filtered, washed, and placed on gradients as above. LP lymphocytes were found in the 70/55 and 55/40 interfaces and comprised 20–30% of the total cells. Cell viability was 74%.

Controls consisted of lymphocytes isolated from bovine spleen but processed according to protocols for the isolation of LP lymphocytes and IEL.

**Lymphocyte proliferation**

Cells were plated on 96-well Linbro plates at a concentration of 0.5 or 1 × 10⁶ cells/well. All cultures were run in triplicate. Cultures were incubated at 37 °C, 5% CO₂ for 72 h, pulsed with 1 μCi ³H-thymidine, harvested 24 h later using a semi-automatic harvester (Skatron), and counted in a Beckman scintillation counter. Data are expressed as the arithmetic mean of c.p.m. from triplicate cultures.

**Immunofluorescent staining**

The percentages of B lymphocytes in LP, IEL, and control preparations were determined by cell surface staining for Ig (sIg), while plasma cells were detected by the presence of cytoplasmic Ig (cIg). For surface staining, cells were suspended in 0.5 mg/ml FITC-labeled anti-Ig in PBS–1% normal rabbit serum, incubated on ice for 30 min, washed and smeared on slides. Slides were fixed in 95% ETOH for 20 min, washed, mounted in buffered glycerol and counted. To visualize cIg, unstained cells were smeared and fixed in ethanol. Slides were incubated with labeled reagent for 45 min, washed, postfixed, mounted in buffered glycerol and counted. Fluorescence was visualized with an Olympus microscope, model BH2-RFL. Samples of bovine LP and IEL were isolated and stained on different days. On each occasion, at least 100 cells were counted on each slide, with replicate counts on each slide and two replicate slides to determine counting error. The data are expressed as the mean percentage of cells stained, ± the standard error.
RESULTS

Cell surface and cytoplasmic staining

Bovine LP lymphocytes, IEL and spleen cells, treated according to the protocols used to isolate gut cells, were characterized with respect to cell surface or cytoplasmic expression of immunoglobulin (Table 1). Thirty-nine percent ±5% of the LP lymphocytes were plasma cells as judged by the presence of cytoplasmic immunoglobulin (cIg). Twenty-seven percent of this population stained positively for surface immunoglobulin (sIg) of any isotype. Far fewer IEL stained positively for either sIg or cIg. Less than 1% IEL stained for cIg, and approximately 9% ±5% of the IEL stained for sIg with the fluoresceinated

TABLE 1

Surface and cytoplasmic staining of LP lymphocytes, IEL and control splenic lymphocytes

|            | Surface Ig (%) | Cytoplasmic Ig (%) |
|------------|----------------|-------------------|
| LP         | 27 ± 6         | 39 ± 5            |
| IEL        | 9 ± 5          | < 1               |
| Spleen (IEL)* | 27 ± 4      | ND                |
| Spleen (LP)* | 28 ± 5      | ND                |
| Spleen     | 33 ± 2         | 10 ± 3            |

* Treated according to the protocol for IEL isolation.
* Treated according to the protocol for LP isolation.
ND, Not determined.

Fig. 1. Responses of bovine IEL and LP lymphocytes to stimulation with anti-bovine Ig. Results are expressed as counts ³H-thymidine incorporated per 5 × 10⁶ cells in an 18-24-h period.

Fig. 2. Representative results from six experiments in which IEL and LP lymphocytes were stimulated in vitro with increasing concentrations of Con A.
anti-Ig reagent. Thus the LP contains a much larger proportion of cells of the B lymphocyte lineage, capable of mounting an antibody response, than does the epithelial tissue.

Controls consisted of lymphocytes isolated from bovine spleen but processed according to protocols for the isolation of LP lymphocytes and IEL. The normal spleen cell population contained 33% ± 2% sIg⁺ cells and 10% plasma cells. When these percentages were compared with those obtained for spleen cells processed according to either the protocol for the recovery of LP lymphocytes or that for isolation of IEL, no differences were observed.

**Proliferation**

Bovine LP lymphocytes and IEL were tested for their ability to proliferate in response to stimulation with anti-bovine immunoglobulin (anti-Ig), Con-

![Graph](image1)

**Fig. 3.** Effect of varying concentrations of bovine Con A supernatants on the proliferative response of bovine IEL and LP lymphocytes. Results are representative of three experiments.

![Graph](image2)

**Fig. 4.** Proliferative response of IEL to stimulation with recombinant human interleukin-2. Results are representative of three experiments.

![Graph](image3)

**Fig. 5.** Proliferative response of LP lymphocytes to stimulation with recombinant human IL-2.
canavalin A (Con A), bovine Con A supernatants (bConA sup) and recombinant human interleukin 2 (rhIL-2). A minimal proliferative response was observed following in vitro treatment of IEL with 0–20 μg/ml anti-Ig (Fig. 1), suggesting that the majority of these lymphocytes do not express sIg. The magnitude of the IEL proliferative response to anti-Ig directly correlated with the number of sIg⁺ cells observed by immunofluorescence. In experiments in which there were higher numbers of sIg⁺ cells in the IEL population, probably due to the presence of LP lymphocytes, larger proliferative responses were seen.

Cultures of LP lymphocytes stimulated with the same concentration of anti-Ig did proliferate, incorporating approximately 40 000 c.p.m. of ³H-thymidine/5×10⁵ cells in an 18–24-h period (Fig. 1). Both lamina propria lymphocytes and IEL responded to Con A stimulation. Data representative of results from six experiments are shown in Fig. 2. Although the magnitude of the responses varied from run to run, peak proliferation was always observed at a Con A concentration of 0.25–1.75 μg/ml. Furthermore, on average, the magnitude of the peak response of LP lymphocytes was 2–3 times that observed for IEL.

LP lymphocytes and IEL were placed in culture with serial dilutions of cell-free supernatants from cultures of Con A-stimulated bovine peripheral blood mononuclear cells. Representative results from three experiments are shown in Fig. 3. Although proliferation of IEL occurred in the presence of these supernatants, proliferation was more marked in cultures of LP lymphocytes. The peak response in both cases occurred in cultures containing 20% supernatant. The results suggest that a higher percentage of LP lymphocytes than IEL are sensitive to stimulation with lymphokines contained in Con A supernatants, although in each case, the cells that do respond have similar sensitivities to the lymphokines, or to inhibitors in the Con A supernatant.

Since both lymphocyte populations responded to stimulation with bovine Con A supernatants which are known to contain a variety of lymphokines, including IL-2, IEL and LP cultures were tested for their ability to proliferate following in vitro stimulation with a rhIL-2 (Biogen). Representative results from five experiments are shown in Fig. 4. A great deal of variation between animals in the magnitude of the peak response was observed. In all cases, however, bovine IEL did respond to rhIL-2, with the peak response occurring at approximately 0.05–0.10 μg/ml. LP lymphocytes also proliferated when cultured with rhIL-2 (Fig. 5), but the concentration needed to give peak stimulation was only 0.01–0.02 μg/ml, or approximately ten-fold less than was needed for peak stimulation of IEL. The results suggest the rhIL-2 and IL-2 in bovine Con A supernatants are capable of influencing proliferative responses of bovine gut mucosal lymphocytes, but that the sensitivities of IEL and LP lymphocytes to IL-2 alone, in the absence of the other lymphokines found in Con A supernatants, may be different.

Again, spleen cells processed according to the protocols for recovery of lym-
phocytes from the LP and epithelium served as controls to demonstrate that
the separation technique has no effect on Con A, bConA sup, anti-Ig, and rhIL-
2 stimulated proliferation. Peak stimulation occurred at 1–5 μg/ml Con A,
0.002–0.0075 μg/ml rhIL-2, and at 20–40% Con A supernatant, whether the
spleen cells were untreated or treated according to the protocols for obtaining
LP lymphocytes, or IEL. In addition, the magnitude of the peak responses for
all treatments was approximately equivalent (100 000–150 000 c.p.m.).

DISCUSSION

Staining patterns observed for bovine gut tissues closely resemble those re-
ported for other species. Parrott et al. (1983) reported that approximately 37%
of LP lymphocytes from the mouse express sIg. This value is somewhat higher
than the 31% reported for cells in the LP of the rat (Lyscom and Brueton,
1982). These values approximate the 27% value we observed for bovine LP.
We consistently observed 39% ± 5% cIg⁺ cells in fixed smears of LP lympho-
cytes. This is in contrast with the observations of Lyscom and Brueton (1982)
who found that only 2% of LP lymphocytes from the rat were plasma cells.
The reason for this difference is unknown. It is possible that this large species
difference exists, or the discrepancy may reflect different histories of antigen
exposure in the two populations. It may also be the result of the different
mononuclear cell isolation procedures utilized.

The degree of cross contamination of IEL with LP mononuclear cells during
the separation process was assessed using fluorescent staining and prolifera-
tion following anti-Ig treatment. Generally, less than 1% of IEL contain cy-
toplasmic immunoglobulin, although in one case, as many as 7% of the cells
recovered from the epithelial layer stained. IEL, isolated at the same time and
cultured with anti-Ig, proliferated more than usual, suggesting that the IEL
population was contaminated during the isolation procedure with cells from
the lamina propria. Contamination of the lamina propria preparation with cells
from the epithelium was assessed by inspection for the presence of epithelial
cells. Using the isolation techniques described, cross contamination was found
to be minimal.

Rodent IEL are reported to consist of T lymphocytes including cytotoxic T
cells (Davies and Parrott, 1981b; Parrott et al., 1983), NK cells (Arnaud-Batt-
tandier et al., 1978; Flexman et al., 1983), cytotoxic cells mediating ADCC
(Nencioni et al., 1983), and a population of Thy 1⁻, Lyt 2⁺ lymphocytes (Petit
et al., 1985). Parrott et al. (1983) reported that only 7% of murine IEL stained
positively for sIg. Even fewer murine IEL stain for cytoplasmic Ig (Lyscom
and Brueton, 1982; Ernst et al., 1985). The low levels of sIg⁺ and cIg⁺ cells
observed in these experiments demonstrate that bovine IEL also contain few
lymphocytes of the B cell lineage.

Antibodies to phenotypic markers associated with functional subsets of bo-
vine T lymphocytes are not widely available. Therefore, functional characteristics of bovine gut cells were assessed. One such characteristic is the ability of cells bearing sIg to proliferate in response to soluble anti-immunoglobulin (Boyd et al., 1985). According to these authors, approximately 40% of murine splenic B cells incorporate $^3$H-thymidine following anti-Ig stimulation, while T cells and non-lymphoid cells do not respond (Boyd et al., 1986). In the present experiments, LP but not IEL responded to anti-Ig. This finding was in agreement with the immunofluorescence data which showed few sIg$^+$ cells in the IEL population.

IEL from different animal species respond differently to T cell mitogens. Guinea pig (Arnaud-Battandier and Nelson, 1982) and pig (Wilson et al., 1986a) IEL proliferate well following Con A stimulation, with maximal proliferation of swine cells occurring at a Con A concentration of 10 $\mu$g/ml. Mouse IEL respond only marginally to Con A treatment (Dillon and MacDonald, 1984) while IEL from the rat large intestine reportedly do not respond at all (Nauss et al., 1984). Maximal proliferation of bovine IEL occurred at a Con A concentration of 1–5 $\mu$g/ml, or 2–10-fold less than the optimal concentration reported for porcine IEL (Wilson et al., 1986a), although this apparent difference in sensitivity may be due to different culture conditions. Bovine LP lymphocytes, like those from the other species documented in the literature (Arnaud-Battandier and Nelson, 1982; Greenwood et al., 1983; Nauss et al., 1984), proliferated when stimulated with Con A.

Related studies designed to investigate the proliferative response of LP and IEL following treatment with Con A supernatants showed that incubation with these supernatants increased proliferation of lymphocytes from both tissues. In addition to other lymphokines, Con A supernatants are known to contain IL-2, and IL-2 has been shown to act across species. More recently, rhIL-2 has been shown to stimulate bovine and porcine peripheral blood mononuclear cells to divide (Fong and Doyle, 1986). The present experiments demonstrate that rhIL-2 can enhance the proliferative responses of lymphocytes from the epithelium and LP of the bovine gut. However, the ability of gut lymphocytes to produce IL-2 in vitro, as has been shown for human (Greenwood et al., 1983), murine (Arnaud-Battandier and Nelson, 1982; Nauss et al., 1984) and porcine (Wilson et al., 1986b) LP has yet to be determined.

Isolation and culture of cells from bovine LP and IEL provide an excellent system in which to study mononuclear cell populations of the bovine gut and to assess mechanisms of immunity which function at mucosal surfaces. It is clear from these studies that populations of bovine LP lymphocytes and IEL, like those from other species, differ with respect to both phenotypic and functional characteristics. In addition, bovine lymphocytes from these compartments share some characteristics of gut cells from other species. Finally, bovine IEL and LP lymphocytes are now known to be sensitive to lymphokines which have been shown to regulate systemic responses. Using this in vitro system,
studies are in progress to determine if the subsets of bovine gut cells, now defined by parameters of Con A and lymphokine sensitivity, have cytotoxic and natural killer activities which have been shown for subsets of gut lymphocytes from other species.

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