Immunology 1996 **87** 71–79

Bovine γ/δ T-cell proliferation is associated with self-derived molecules constitutively expressed *in vivo* on mononuclear phagocytes

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SUMMARY

Bovine γ/δ T cells have been shown previously to proliferate when cocultured with γ-irradiated bovine monocytes in the 'autologous mixed leucocyte reaction' (AMLR). It was suggested that the response may be to culture-derived or culture-induced antigenic epitopes. Data presented here indicate that the γ/δ T-cell stimulatory activity is attributable to a self-derived cell-surface molecule of mononuclear phagocytes that is constitutively expressed *in vivo*. The ability to induce an AMLR did not require *in vitro* culture or stress associated with *in vitro* isolation of cells or increased temperature since it could be induced by monocytes fixed by paraformaldehyde during blood collection from normal animals. Furthermore, stimulation by monocytes did not depend upon secreted molecules since fixed monocytes that had been incubated overnight at 37° to allow secretion of preformed molecules, or subjected to hypotonic shock in H_2O for 10 min before addition to the cultures, induced an AMLR as did plasma membranes prepared from *ex vivo* monocytes. In contrast, enzymatic treatment of monocytes to digest surface molecules followed by fixation destroyed their ability to stimulate an AMLR. The ability of monocytes to stimulate proliferation of γ/δ T cells was distinguishable from their ability to stimulate α/β T cells, since the former was destroyed by glutaraldehyde fixation whereas stimulation of α/β T cells by major histocompatibility complex (MHC)-presented antigenic epitopes is not. Moreover, induction of proliferation of bovine γ/δ T cells was not MHC-restricted. Finally, bovine alveolar macrophages, sheep monocytes and transformed bovine monocytes stimulated proliferation of bovine γ/δ T cells whereas none of the following did so: human monocytes, murine macrophages, bovine myeloid cells other than mononuclear phagocytes, other nucleated cells found in bovine blood including activated MHC class II-bearing B cells, and a variety of species of bacteria. Thus, the stimulatory epitope is unique to and conserved among mononuclear phagocytes of ruminants. Demonstration of stimulation of bovine γ/δ T cells by self-derived molecules is consistent with reports for murine γ/δ T cells.

INTRODUCTION

While the majority of T lymphocytes in the peripheral blood of adults are α/β T cells that recognize antigenic peptides presented by autologous major histocompatibility complex (MHC) molecules, a second lineage of T lymphocytes known as γ/δ T cells also exists.[1] The γ/δ T cells are found in high proportions at epithelial surfaces in some mammalian species and they predominate in the blood of young ruminants suggesting that they are particularly important for the immune defence of young animals.[1,2] While the antigen-specific receptor of γ/δ T cells is structurally similar to that of α/β T cells, it is coded for by a separate set of genes[3] and in contrast to α/β T cells the antigen specificity of γ/δ T cells and their physiological role is largely an enigma at this time.

γ/δ T cells have been shown to be involved in viral (influenza A virus and Epstein–Barr virus), bacterial (*Mycobacterium* spp., *Brucella melitensis*, *Listeria monocytogenes*, streptococci and staphylococci) and protozoal (*Trypanosoma cruzi* and *Plasmodium falciparum*) infections (for review see refs. 4 and 5), and human and murine γ/δ T lymphocytes are activated by addition of microbial organisms to cultures of mononuclear cells.[6–10] Reports of specificity of γ/δ T cells for infectious microbes, however, have been largely confined to mycobacterial components including heat-shock protein (HSP) as well as a non-proteinaceous mycobacterial substance.[11,12] It is unclear if the γ/δ T-cell responses are to antigenic peptides presented by endogenous molecules such as MHC molecules including the non-classical molecules known to be involved in γ/δ T-cell responses[13,14] or if the responses may be to endogenous or self-derived molecules induced by the presence of the mycobacterial

Received 18 August 1994; revised 22 July 1995; accepted 3 August 1995.

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components (for review see ref. 10). Responses by γ/δ T cells to self-derived antigens have also been reported. Some of these antigens such as HSP may stimulate γ/δ T-cell responses via an epitope that is conserved with bacteria.[15–17] Other examples of self-induced responses include murine epithelia-derived keratinocytes that activate epidermal γ/δ T cells to secrete interleukin-2 (IL-2),[18] and γ/δ T cells from the intestinal epithelial lining of weanling mice that react to an undefined self molecule.[19,20] γ/δ T cells have also been associated with a variety of autoimmune diseases such as rheumatoid arthritis,[21] pulmonary sarcoidosis[22] and polymyositis.[23]

The large percentage of γ/δ T cells found in the circulation of young ruminants[1] and the expression of a γ/δ T-cell lineage-specific cell-surface molecule known as T19 or WC1[24,25] make cattle a useful model for elucidating the role of γ/δ T cells *in vivo*. It has been shown that bovine γ/δ T cells proliferate when cocultured with autologous γ-irradiated monocytes without requiring the addition of exogenous antigens in the 'autologous mixed leucocyte reaction' (AMLR).[25,26] It was suggested in the initial study of the AMLR response in cattle that the stimulator cells may require changes in expression of MHC or costimulatory molecules during the *in vitro* culture to effectively stimulate γ/δ T-cell proliferation. This was partly based on the observation that glutaraldehyde-fixed *ex vivo* monocytes did not stimulate γ/δ T cell proliferation.[26] Here we present evidence that the stimulatory property of monocytes is due to a self-derived component constitutively expressed *in vivo*.

MATERIALS AND METHODS

Animals

Blood donors were female *Bos taurus* Holsteins, 2 years of age. Cattle were kept in an open holding pen.

Isolation of peripheral blood mononuclear cells (PBMC)

Blood was collected by venepuncture of the jugular vein either into a solution of heparin or it was defibrinated as described previously.[26] For some experiments the blood was collected into an equal volume of a 2% solution of paraformaldehyde in phosphate-buffered saline (PBS) that also contained heparin. PBMC were isolated by density gradient centrifugation of blood over Ficoll–Hypaque (Ficoll–Paque, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) using standard techniques. Mononuclear cell populations isolated from defibrinated blood, which contain fewer monocytes than those from heparinized blood,[27] were further depleted of monocytes by incubating the cells in polystyrene tissue culture flasks for 1 hr at 37° in an atmosphere of 5% CO_2 in air and are referred to as monocyte-depleted PBMC (MD-PBMC). PBMC isolated from blood collected in heparin are referred to simply as 'PBMC'.

Isolation of monocytes

Monocytes were isolated from PBMC by adherence to plasma-coated gelatine as described previously.[27]

Other cell populations assessed as stimulator cells

The following cell lines were maintained as continuous cultures at 37° in an atmosphere of 5% CO_2 in air. BL3, a bovine leukaemia line,[28] and K562, a human myelogenous leukaemia (American Type Culture Collection; ATCC), were maintained in Sigma Hybrimax medium (Sigma Chemical Company, St Louis, MO) with 60 μg/ml gentamicin. J774A.1, a transformed mouse monocyte line; L929, a mouse fibroblast line; Madin–Darby bovine kidney fibroblasts (MDBK); and D17, a cell line derived from a canine osteogenic sarcoma were obtained from ATCC and maintained in complete RPMI (cRPMI: RPMI-1640 with 10% heat-inactivated fetal calf serum (FCS), 2 μM L-glutamine, 60 μg/ml gentamicin, 5×10^{-5} M 2-mercaptoethanol). M167, a transformed bovine monocyte cell line,[29] was maintained in Dulbecco's minimum essential medium (MEM) with 10% heat-inactivated FCS, 60 μg/ml gentamicin and 5×10^{-5} M 2-mercaptoethanol. Alveolar macrophages were obtained from the lavage fluid of allogeneic Holstein calves and monocytes were isolated from the peripheral blood of the same animal. Activated MD-PBMC were obtained by stimulation with pokeweed mitogen (PWM; Sigma) at 1 μg/ml in standard cultures. Cells to be added to AMLR cultures as stimulators were γ-irradiated 24 hr after activation. An aliquot of the PWM-stimulated cells was allowed to proceed in the culture and pulsed with [^{3}H]thymidine to ensure that activation occurred. Assessment of the stimulated cultures by indirect immunofluorescence assay (IFA) revealed 75% B cells, 17% γ/δ T cells and 18% α/β T cells. BL3 cells were similarly activated with PWM and activation was assessed by acquisition of surface immunoglobulin as assessed by IFA with monoclonal antibody (mAb) IL-A58.[30] Granulocytes were isolated from peripheral blood by collecting the cells that went through the Ficoll–Hypaque layer of the density gradient and lysing the erythrocytes by hypotonic shock. The population contained approximately equal numbers of neutrophils and eosinophils.

Bacteria

The following bacteria were cultured by standard techniques in broth, harvested in log phase of growth and killed by heating at 80° for 30 min, except for *Mycobacterium bovis* which was killed by methanol fixation: *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *M. bovis* (BCG strain), *Brucella abortus* strains 19 and 2308.

Cell fixations

Cells were fixed with 1% paraformaldehyde as previously described by Suttles *et al.*[31] or with glutaraldehyde as described by Schimonkevitz *et al.*[32] In some experiments, fixed cells were assessed for their ability to respond to stimulation by Concanavalin A (Con A) (Sigma) at 1 μg/ml.

Enzymatic treatment of monocytes

PBMC or isolated monocytes were resuspended to 10^7 cells/ml in RPMI-1640. The cells were treated with the following enzymes: 12·5 μg/ml β-*N*-acetylglucosaminidase (Boehringer Mannheim, Indianapolis, IN), 100 μg/ml proteinase K (United States Biochemical, Cleveland, OH), 100 μg/ml α-glucosidase (United States Biochemical), 10 μg/ml neuraminidase (Boehringer Mannheim), 5 μg/ml chymotrypsin (Boehringer Mannheim), or 5 μg/ml trypsin (Gibco, Gaithersburg, MD). The cells in the presence of the enzyme were incubated for 1 hr at 37°and shaken every 15 min. After incubation, the cells were washed three times in PBS and enzymatically-treated cells were either paraformaldehyde-fixed or held in cRPMI on ice until addition to cultures.

Preparation of cell membranes

Membranes were prepared from monocytes, MD-PBMC or BL3 cells as described by Brian.[33] Briefly, cells were resuspended to between 10^6 and 5×10^6 cells/ml in cold homogenization buffer (20 mM Tris–HCl, 10 mM NaCl, 0·1 mM $MgCl_2$, 0·1 mM phenylmethylsulphonyl fluoride, 0·5 μg Dnase I/ml), frozen and thawed three times and homogenized for 3 min at speed 5 on a Beckman cell homogenizer to disrupt >90% of the cells. The homogenate was underlaid with 41% sucrose in homogenization buffer and centrifuged at 95 000 *g* for 1 hr. The cytoplasmic membrane layer was removed and washed twice in RPMI-1640 by centrifugation at 115 000 *g* for 30 min. The remaining homogenate in the 41% sucrose was diluted fivefold in RPMI-1640 and washed twice by centrifugation at 115 000 *g* for 30 min to isolate microsomal and accompanying cytosolic components of the cells. The various fractionated components were then resuspended in medium and added to cultures of MD-PBMC at a concentration equivalent to that of the intact monocytes in the AMLR.

Autologous mixed leucocyte cultures

AMLR cultures were established in 96-well flat bottom microtitre plates using 5×10^6 MD-PBMC per well as responder cells as described previously[26] in either cRPMI or Hybrimax serum/protein-free medium (Sigma) supplemented with 5×10^{-5} M 2-mercaptoethanol and 60 μg/ml gentamicin. Stimulator cells in standard assays were either PBMC which had been collected in heparin and contained 7–15% monocytes or they were populations enriched for monocytes. As indicated, stimulator cells received 5000 Rad of γ-irradiation from a ^{137}Cs source or were fixed with paraformaldehyde or glataraldehyde as described above prior to addition to the AMLR cultures. Where indicated, cells other than autologous γ-irradiated PBMC or monocytes were evaluated as stimulators of γ/δ T-cell proliferation. These included other mammalian and bacterial cells as well as cell membranes. Cultures contained a total volume of 200 μl per well and were maintained in a humidified atmosphere at 37° with 5% CO_2 in air. Control cultures which did not receive stimulator cells were prepared for each experiment to assess background levels of proliferation. After 5 days of culture the amount of cell proliferation was measured by incubating the cells overnight with 0·5 μCi of [^{3}H]thymidine per culture well. The incorporated radioactivity was assessed by liquid scintillation. Triplicate cultures were established and the mean ± SD of c.p.m. of incorporated radioactivity was calculated for each treatment. In some experiments, control cultures of Con A-stimulated cells were also established using Con A at 1 μg/ml.

Indirect immunofluorescence

IFA was conducted using cell-type-specific mAb and analysed by flow cytometry or ultraviolet microscopy as previously described.[34] The following mAb were used: IL-A29 (IgG1), reacts with the bovine γ/δ T-cell WC1 surface protein;[25] IL-A12 reacts with bovine CD4;[34] IL-A51 reacts with bovine CD8;[35] IL-A15 reacts with bovine; IL-A58 reacts with bovine immunoglobulin λ and κ light chains;[30] CACTB6A (IgM) and CACTB81A (IgG1) react with the N6 and N7 epitopes of the γ/δ TcR, respectively (VMRD Inc., Pullman, WA). To assess blasting cells, cells in the AMLR were size gated by comparison with non-stimulated control cultures of MD-PBMC and analysed for expression of lineage-specific cell-surface antigens by flow cytometry.

Enrichment for ex vivo *γ/δ T cells*

MD-PBMC were enriched to between 93 and 97 ± 3% γ/δ T cells by negative selection over a mouse T-cell immunocolumn (Biotex Laboratories, Edmonton, Canada) essentially as described by the manufacturer. To remove non-γ/δ T cells including α/β T cells, B cells and residual monocytes, MD-PBMC were incubated with a cocktail of mAb IL-A12, IL-A51, IL-A42 (anti-bovine CD2; ATCC), IL-A58, and CACT83B and CACT138A (VMRD; anti-bovine CD4). Cells were incubated with the cocktail and passaged over the column twice, using the same column for both passages with extensive washing with PBS between the first and final run.

Table 1. Comparison of treatments of stimulator cells in the AMLR

	Treatment of stimulator cells for AMLR			
Exp. no.*	Medium	γ-Irradiated	Paraformaldehyde-fixed	Glutaraldehyde-fixed
1	3587 ± 577†	21 635 ± 7338	27 266 ± 5998	1690 ± 236
2	1906 ± 1577	14 233 ± 1308	19 556 ± 3769	2091 ± 132
3	3110 ± 2402	17 244 ± 5951	28 054 ± 4449	3946 ± 763
4	497 ± 195	49 827 ± 8185	66 541 ± 13 977	606 ± 609

*Exp. 2 was performed using PBMC as stimulator cells; the other experiments used monocytes as stimulator cells. Exp. 1 was performed with cells from animal #65; others cells were from #66. Responder cells were MD-PBMC in all experiments.

†Mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in triplicate cultures is indicated. There was no significant difference ($P > 0.05$) in the level of proliferation in cultures with γ-irradiated stimulator cells and those with paraformaldehyde-fixed stimulator cells as determined by the Mann–Whitney *U* test, whereas there was significantly less proliferation in cultures with glutaraldehyde-fixed stimulator cells ($P \leq 0.001$).

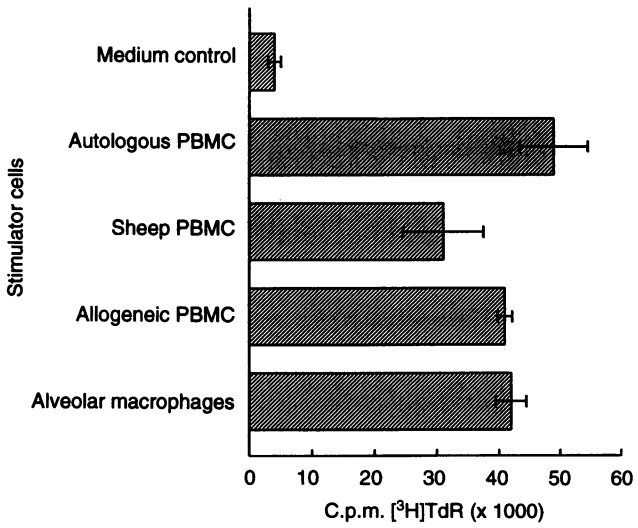

Figure 1. Proliferative response of *ex vivo* γ/δ T cells (purified by affinity column to 93–97% with a SD of 3% based upon reactivity with mAb IL-A29) following stimulation with the various γ-irradiated cell populations indicated. Proliferation was measured by incorporation of [^{3}H]thymidine. The response indicated is the mean ± SD of triplicate cultures.

RESULTS

Stimulation of γ/δ T cell proliferation in the AMLR

Proliferation of bovine MD-PBMC occurs when cells are cocultured with γ-irradiated autologous monocytes in the AMLR.[26] In our studies approximately 93% of the blasting cells in the AMLR were WC1$^+$, a lineage-specific marker of bovine γ/δ T cells. Here we showed that the AMLR occurred when conducted in either cRPMI or Hybrimax serum-free/protein-free medium (data not shown), indicating that it was not a response to serum proteins contributed by the addition of FCS, results similar to those reported previously.[26] In addition, we showed that monocytes fixed with 1% paraformaldehyde after isolation from blood but before addition to AMLR cultures induced proliferation equivalent to that induced by γ-irradiated PBMC suggesting the stimulatory molecule was not culture-acquired or a result of *in vitro* activation (Table 1). In contrast, glutaraldehyde-fixed stimulator cells did not induce proliferation (Table 1), concurring with a previous report.[26] The results suggest that the lack of stimulation by glutaraldehyde-fixed stimulators is a result of destruction of the stimulatory epitope by glutaraldehyde rather than its absence from *ex vivo* monocytes. Moreover, they suggest that the stimulatory molecule differs from presentation of peptides by MHC-molecules to α/β T cells, since glutaraldehyde fixation of antigen-presenting cells has been shown elsewhere to preserve their ability to stimulate responses by murine[32] and bovine [36] α/β T cells. In keeping with this, allogeneic monocytes induced proliferation of MD-PBMC of purified γ/δ T cells (Fig. 1) confirming results from previous studies that the ability to induce γ/δ T-cell proliferation is not MHC-restricted.[26]

Stimulatory capacity is associated with the membrane of *ex vivo* monocytes

To ensure that expression of the stimulatory molecule or epitope did not result from stress associated with the cell isolation procedure, cells were fixed with paraformaldehyde coincident with collection from the jugular vein. These cells induced proliferation in the AMLR, as did cells fixed at various stages of the isolation procedure (Table 2). PBMC isolated from blood collected in paraformaldehyde did not proliferate to stimulation with Con A, indicating that they were adequately fixed (data not shown). Fixed monocytes subjected to hypotonic shock in dH_2O for 10 min before addition to the AMLR cultures, and thus almost certainly metabolically inactive, also retained the ability to induce proliferation in the AMLR (8645 ± 1463 c.p.m.) to levels similar to that induced by γ-irradiated PBMC (10 347 ± 3472 c.p.m.; medium control cultures without stimulator cells had 1007 ± 458 c.p.m.).

Since treatment with 1% paraformaldehyde does not inhibit the secretion of preformed molecules,[31] paraformaldehyde-fixed stimulator cells were incubated overnight at 37°

Table 2. Comparison of paraformaldehyde fixation at different points during the isolation of stimulator cells

		Treatment of stimulator cells added			
Exp. no.*	No stimulators	γ-Irradiated	Fixed in the blood†	Fixed after isolation‡	Fixed after γ-irradiation§
1	1279 ± 162¶	30 282 ± 19 670	37 604 ± 3526	ND	ND
2	1406 ± 381	30 297 ± 4365	62 207 ± 5467	ND	ND
3	406 ± 94	28 070 ± 5511	16 570 ± 4192	13 571 ± 9411	11 128 ± 823
4	1831 ± 1509	14 038 ± 2511	13 393 ± 5194	6534 ± 289	12 351 ± 1285
5	2877 ± 764	73 346 ± 5125	ND	28 249 ± 2927	21 326 ± 7186

*Exp. 1, 2 and 4 were performed with cells from animal #66, Exp. 3 and 5 with cells from #65. Responder cells were MD-PBMC for all experiments.

†PBMC were fixed with paraformaldehyde coincident with collection of blood.

‡PBMC were fixed after isolation by Ficoll–Hypaque density gradient centrifugation.

§PBMC were fixed after isolation and γ-irradiation.

¶Mean ± SD of c.p.m. of [^{3}H]thymidine incorporated in replicate cultures. ND, not done.

Table 3. Evaluation of membranes as stimulators for γ/δ T-cell proliferation

Exp. no.*	No stimulators	γ-Irradiated PBMC	Membranes from: Monocytes	BL3 cells	Monocyte-depleted PBMC
1	1500 ± 948†	36799 ± 3934	12955 ± 5945	1377 ± 320	ND
2	1820 ± 586	8342 ± 1203	9226 ± 1142	1936 ± 574	ND
3	376 ± 90	43848 ± 3469	20932 ± 2095	523 ± 140	ND
4	463 ± 53	39223 ± 4744	21659 ± 9560	ND	325 ± 147
5	907 ± 100	2859 ± 209	6021 ± 405	ND	996 ± 777

*Exp. 1 and 4 were performed with cells from animal #66, Exp. 2, 3 and 5 with cells from #65. Responder cells were MD-PBMC in all experiments.

†Mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in triplicate cultures. ND, not done. There was no significant difference in the proliferation induced by γ-irradiated PBMC and the monocyte membranes ($P > 0.05$) as determined by the Mann–Whitney U test. There was significantly less proliferation in response to stimulation with membranes from BL3 or monocyte-depleted PBMC relative to that induced by γ-irradiated PBMC ($P \leq 0.001$).

before use to allow secretion of potential soluble stimulatory molecules. Addition of cells treated in this way to cultures of MD-PBMC induced proliferation (27849 ± 4815 c.p.m.) to levels comparable to that achieved in the AMLR when γ-irradiated PBMC were used as the stimulator cells (29411 ± 5623 c.p.m.). To confirm the association of the stimulatory property with the cell membranes of monocytes, membranes were isolated from monocytes. We found that membranes substituted for intact monocytes for induction of proliferation in the AMLR (Table 3). In contrast, control membrane preparations from BL3 cells or from MD-PBMC did not (Table 3), nor did cytosolic/microsomal components of the monocytes (1428 ± 714 c.p.m. in cultures stimulated with cytosolic/microsomal components compared to 8180 ± 1442 c.p.m. in cultures stimulated with γ-irradiated PBMC and 451 ± 243 c.p.m. in medium control cultures). Finally, we showed that γ-irradiated monocytes that had been subjected to various enzymatic treatments (trypsin and chymotrypsin to remove proteins from the monocyte cell surface; α-glucosidase to remove sucrose and mannose residues from glycoproteins; and neuraminidase to hydrolyse the linkage joining a terminal sialic acid residue to a D-galactose of a D-galactosamine thus removing the moieties from cell surface glycoproteins) followed immediately by paraformaldehyde fixation were unable to stimulate an AMLR (Table 4). However, when monocytes were enzymatically treated and then added to the cultures without fixation, γ/δ T-cell proliferation occurred indicating that the stimulatory molecule could be re-expressed during the culture period and that stimulator cells were not irrevocably damaged by the enzymatic procedures (Table 4). The results from treatment with β-*N*-acetylglucosaminidase and proteinase K were similar to those from the treatment with trypsin and chymotrypsin and therefore not shown.

The stimulatory factor is conserved among mononuclear phagocytes of ruminants and following transformation

Further experiments evaluated conservation of the stimulatory ability among mononuclear phagocytes. While both human PBMC containing approximately 10% monocytes and the

Table 4. Effect of various enzymatic treatments on the ability of stimulator cells to induce an AMLR

Enzymatic treatment	Target of enzyme	Post-enzymatic treatment* γ-Irradiated	Paraformaldehyde-fixed
None	—	55082 ± 6838†	64847 ± 5688
Neuraminidase	Sialic Acid	71000 ± 7975	493 ± 138
Chymotrypsin	Trp, Phe, Tyr	62775 ± 5043	743 ± 122
Trypsin	Lys, Arg	64077 ± 2707	1680 ± 1395
α-glucosidase	Mannose, Sucrose	56683 ± 1361	542 ± 419

*Stimulator cells were enzymatically treated as indicated and then either γ-irradiated or paraformaldehyde-fixed and added to the AMLR. Responder cells were MD-PBMC in all experiments.

†Mean c.p.m. ± SD of [^{3}H]thymidine incorporation in triplicate cultures. The experiment was performed twice with similar results. Background c.p.m. in medium control cultures with no stimulator cells added was 646 ± 226. There was significantly less proliferation in cultures stimulated with cells that were paraformaldehyde-fixed after enzymatic treatment compared to those γ-irradiated after treatment ($P \leq 0.001$).

Table 5. Evaluation of various mononuclear phagocytes as stimulators of γ/δ T-cell proliferation

Stimulator cells used*	Stimulator cell	Exp. 1	Exp. 2
None	γ-irradiated	1608 ± 1506†	886 ± 698
Auto PBMC		71 593 ± 18 882	79 516 ± 14 928
Human PBMC		1926 ± 2213	488 ± 68
None	γ-irradiated	7350 ± 1693	969 ± 350
Auto PBMC		60 389 ± 4368	42 368 ± 8634
Murine M0 J774A.1		428 ± 197	540 ± 58
None	γ-irradiated	437 ± 148	ND
Auto PBMC		61 027 ± 3928	
Allo PBMC		56 925 ± 1634	
Sheep PBMC (#700)		40 449 ± 2526	
Sheep PBMC (#841)		34 954 ± 8271	
Alveolar bovine M0		44 885 ± 2004	
None	γ-irradiated	283 ± 149	186 ± 72
Auto PBMC		21 645 ± 4172	2906 ± 663
Bovine trnsf′ M0 (M167)		9100 ± 553	1995 ± 108
None	Para-fixed	1667 ± 1697	827 ± 341
Au PBMC		71 754 ± 11 693	62 215 ± 4013
Murine M0 J774A.1		386 ± 97	379 ± 92

*Auto, autologous; Allo, allogeneic; M0, macrophage; Para-fixed, paraformaldehyde-fixed; transf′, transformed. Medium control cultures without stimulator cells are indicated as 'none' under the column 'Stimulator cells'. Responder cells were MD-PBMC in all experiments.

†Mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in triplicate cultures.

mouse macrophage cell line J774A.1 failed to induce proliferation of monocyte-depleted bovine PBMC, PBMC from sheep could do so (Table 5). Immunofluorescence evaluation of cells (Exp. 1, Table 5) after 1 week of culture indicated that stimulation with γ-irradiated sheep PBMC resulted in an increase in the proportion of γ/δ T cells to 61%, from approximately 29% in the starting population. By comparison, in control cultures stimulated with autologous γ-irradiated PBMC, the proportion of γ/δ T cells was increased to 74%. Bovine alveolar macrophages also stimulated bovine MD-PBMC to proliferate (Table 5), and both bovine alveolar macrophages and sheep PBMC stimulated proliferation of purified *ex vivo* γ/δ T cells (Fig. 1). Similarly, the transformed bovine monocyte line M167 also stimulated proliferation of MD-PBMC (Table 5), although the level of proliferation using M167 as stimulator cells was not as great as that in cultures stimulated with γ-irradiated autologous PBMC. Nevertheless, stimulation by the M167 cell line induced an increase in γ/δ T cells to 56%, comparable to the 59% in control cultures stimulated with γ-irradiated autologous PBMC (Exp. 1, Table 5).

Stimulatory property is restricted to mononuclear phagocytes

None of the cells associated with bovine blood outwith monocytes stimulated proliferation of γ/δ T cells in cultures of MD-PBMC (Table 6). Those evaluated included other cells of the myeloid lineage (granulocytes); fibroblasts (bovine MDBK and murine L929); and lymphocyte populations, including class II-bearing activated lymphocytes and the transformed bovine B cell line BL3. Although γ/δ T cells have been reported to be cytolytic for natural killer (NK) cells (see ref. 10 for review), the cell lines K562 and D17, NK target cells recognized by lymphokine-activated bovine killer cells,[37] did not stimulate proliferation of γ/δ T cells (Table 6). In some cases, paraformaldehyde fixation was employed to preserve the integrity of short-lived cells such as granulocytes.

Lack of conservation of the stimulatory molecule with prokaryotic cells

To determine if the ability to stimulate bovine γ/δ T-cell proliferation was conserved between monocytes and prokaryotic cells as a result of a common epitope, as shown for the HSP 60 epitope shared between human mononuclear phagocytes and mycobacteria[15] or that shared with between bacteria and transformed human B cells,[16] we evaluated the ability of bacteria to induce proliferation of MD-PBMC or PBMC with monocytes present to process antigens and provide other accessory functions. No proliferation of γ/δ T cells was detected in either type of culture with any of the five bacteria tested. Addition of bacteria to AMLR cultures or cultures of responder cells stimulated with Con A also did not augment proliferation. Rather in some instances, addition of bacteria to AMLR cultures decreased proliferation without affecting Con A-induced proliferation (data not shown).

DISCUSSION

Although the inability of glutaraldehyde-fixed monocytes to

Table 6. Evaluation of various cell types as stimulators of γ/δ T-cell proliferation*

Stimulator cells used	Stimulator cells	Exp. 1	Exp. 2
None	γ-irradiated	437 ± 148	908 ± 100
Auto PBMC		61 027 ± 3928	1940 ± 267
Act'ed lymphocytes		679 ± 132	712 ± 394
None	γ-irradiated	1075 ± 445	ND
Auto PBMC		11 641 ± 2301	
Act'ed lymphocytes		2694 ± 1456	
Act'ed BL3		1467 ± 530	
None	γ-irradiated	969 ± 350	7350 ± 1693
Auto PBMC		42 368 ± 8634	60 389 ± 4368
MDBK		396 ± 79	457 ± 228
L929		273 ± 175	427 ± 16
K562		635 ± 57	828 ± 149
None	γ-irradiated	2576 ± 1033	1515 ± 576
Auto PBMC		45 555 ± 1735	43 116 ± 7012
MDBK		673 ± 134	336 ± 263
BL3		466 ± 63	721 ± 105
None	γ-irradiated	273 ± 89	589 ± 63
Auto PBMC		2048 ± 163	3962 ± 243
D17		417 ± 72	638 ± 147
None	Para-fixed	2576 ± 1033	422 ± 56
Auto PBMC		45 555 ± 1735	10 938 ± 1375
Au granulocytes		878 ± 311	759 ± 123
MDBK		717 ± 444	1069 ± 481
None	Para-fixed	1667 ± 1697	827 ± 341
Auto PBMC		71 754 ± 11 693	62 215 ± 4013
L929		472 ± 160	367 ± 67
BL3		1287 ± 108	1207 ± 129
K562		1874 ± 401	2983 ± 1422

*Auto, autologous; Para-fixed, paraformaldehyde-fixed; act'ed lymphocytes, PWM-activated lymphocytes; none, medium control cultures without stimulators. Data indicated are mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in triplicate cultures. Responder cells were MD-PBMC in all experiments.

stimulate γ/δ T-cell proliferation in the AMLR was interpreted in a previous study to suggest that either the monocytes had to be metabolically active in order to produce or express the stimulatory or costimulatory molecule(s)[26] or that the monocyte stimulatory ability was a culture-acquired property.[26] The results presented here suggest that this is not the case since paraformaldehyde-fixed *ex vivo* PBMC and membranes from *ex vivo* monocytes stimulated an AMLR. Moreover, while murine and human γ/δ T cells have been shown to respond to stress-induced heat-shock proteins,[11,17] our experiments ruled out the possibility that the monocyte stimulatory molecule(s) was induced in response to the stress of *in vitro* manipulations since monocytes fixed during collection of blood stimulated an AMLR. These results suggest that the stimulatory molecule is expressed constitutively *in vivo*. However, we are unable to rule out the rapid induction of the stimulatory molecule *in vivo* caused by the stress of animal restraint and venepuncture, although it should be noted that the experimental animals employed willingly submit to this procedure.

While experiments reported here clearly showed that the stimulatory ability of monocytes was associated with the monocyte membrane, the AMLR stimulatory activity was distinguishable from peptide presentation to α/β T cells on MHC molecules based on the fact that the response was not MHC-restricted nor induced by glutaraldehyde-fixed stimulator cells. It is likely that glutaraldehyde fixation destroyed the γ/δ T-cell stimulatory moiety of the monocytes directly rather than prevented expression of costimulatory molecules since results from our studies with monocyte membranes argue against the need for induction of costimulatory molecules on the monocytes. We are assuming that the ability of monocytes to induce proliferation of γ/δ T cells in the AMLR is a result of a direct interaction between the two cell types. This is supported by the fact that γ/δ T-cell lines consisting of 84% IL-A29$^+$ cells as well as populations of *ex vivo* MD-PBMC enriched to 93–97% IL-A29$^+$ cells proliferate in response to stimulation with γ-irradiated monocytes, although they may require addition of exogenous IL-2 (here and M. Hanby-Flarida *et al.*, submitted for publication). We cannot discern from our studies whether the stimulatory molecule is an integral membrane protein or a presented molecule, although there is an increasing amount of evidence for stimulation of γ/δ T cells by non-presented

molecules (for review see ref. 38). Moreover, it is unclear whether the enzymatic treatments directly affected the stimulatory molecule(s), suggesting that it is a sialylated glycoprotein based upon the fact that proteases, endoglycosylases and neuraminidase all destroyed the ability of monocytes to stimulate proliferation or if the lack of response was due in part to degradation of adhesion or costimulatory molecules on the monocytes. Little is known about the role of accessory molecules in γ/δ T cells although it has been shown that the vitronectin receptor is important for activation of murine γ/δ T cells.[39]

Results presented here concur with descriptions of some other autoreactive γ/δ T cells. That is the stimulator cells are in the same tissue as the responsive γ/δ T-cell population[18,19] and proliferation of murine γ/δ T cells induced by keratinocytes also is not MHC-restricted.[18] Our results also indicated, however, the stimulatory property was not restricted to mononuclear phagocytes in peripheral blood. We have no information regarding the converse situation, i.e. the ability of blood monocytes to stimulate γ/δ T cells from other tissues.

The inability of activated B cells to stimulate proliferation of γ/δ T cells indicates that MHC class II expression alone is not sufficient nor does it facilitate expression of a stimulatory capacity through its ability to present self-derived peptides. Further, the inability of bacteria to stimulate suggests the molecule responsible for stimulating bovine peripheral blood γ/δ T cells is likely to differ from the epitope shared between transformed human B cells and bacteria that are responsible for stimulating a subpopulation of human peripheral blood γ/δ T cells[16] and that bovine γ/δ T cells differ from human γ/δ T-cell subpopulations.[6,7] It should be borne in mind that it may be necessary to have mononuclear phagocytes present in the culture system to present bacterial antigens or provide monokines for 'bacterial antigen-induced' responses to occur. Such experiments are difficult to perform in the bovine system since γ-irradiated mononuclear phagocytes stimulate proliferation of bovine γ/δ T cells and non-irradiated monocytes suppress proliferation.[26] We attempted to evaluate this by adding bacteria to AMLR cultures that therefore contained γ-irradiated monocytes or cultures of PBMC with non-irradiated monocytes present, but still found no induction of or increase in proliferation. It is possible that monocytes do not express the appropriate restriction element for presentation of such antigens.

The γ/δ T cell-stimulating element of bovine mononuclear phagocytes may be homologous/analogous to the γ/δ T cell-stimulating elements in humans and mice that are postulated to be self-derived but whose expression in some instances is induced by microbes (see ref. 10) or in others is expressed by autologous cells at particular differentiation states.[40] The bovine model may largely differ from the latter ones by virtue of the lack of necessity to be induced, i.e. its apparently constitutive expression. The question arises then, 'why are the γ/δ T cells not stimulated constitutively *in vivo*?' It has also been shown previously that *ex vivo* monocytes prevent proliferation of γ/δ T cells if the monocytes constitute > 10% of the total responder cell population and have not been subjected to γ-irradiation.[26] We hypothesize that when monocytes become damaged (as exemplified by γ-irradiation or paraformaldehyde fixation) the monocyte regulatory/inhibitory function ceases to be expressed and the γ/δ T cells proliferate in response to the constitutively expressed stimulatory molecule. It has been suggested that a possible role for autoreactive T cells is to augment antigen-specific T-cell responses by cytokine production.[40,41] In the model we have proposed, γ/δ T cells could respond during an inflammatory response or infection and secrete cytokines independent of the foreign peptides presented by MHC molecules. This would presumably be one of the earliest immune responses to cellular damage. Our hypothesis is supported by the fact that M167, a transformed bovine monocyte cell line, retained the ability to stimulate proliferation of γ/δ T cells.

ACKNOWLEDGMENTS

Funding for the work was provided by American Cancer Society Grant #IRG-16-30 and USDA Competitive Grant Award #91-02363 to C.L.B.

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