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Mechanisms of Resistance to Herpesviruses: Comparison of the Effectiveness of Different Cell Types in Mediating Antibody-Dependent Cell-Mediated Cytotoxicity

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Enriched populations of polymorphonuclear leukocytes (PMN) and macrophages obtained from the mammary gland and of granulocytes (PBG) and lymphocytes (PBL) prepared from peripheral blood of the same animal were compared for their ability to mediate antibody-dependent cell cytotoxicity against antibody-sensitized infectious bovine rhinotracheitis virus-infected target cells (IBR-GBK) and antibody-sensitized chicken erythrocyte targets (CRBC). The order of effectiveness was PMN > macrophages > PBG > PBL. The reason why PBG (86% PMN) were less than 50% as active as mammary PMN (99% PMN) was explored and discussed. The findings that PMN were more effective on a cell-to-cell basis, required less antiserum to sensitize for cytotoxicity, and destroyed IBR-GBK cells faster and more completely than other cell types could mean that PMN may be the cell type most important in causing early recovery from herpesvirus infections.

Although it still remains to be proven, many have speculated that antibody-dependent cell-mediated cytotoxicity (ADCC) represents an in vitro model for antiviral defense mechanisms (12, 16, 21), tumor immunity (4, 8, 17), and the production of some disease states such as autoimmunity (1). Certainly from in vitro studies, the parameter would seem to represent a powerful one since only low concentrations of antisera are needed to sensitize target cells for destruction and a diverse array of cell types can mediate the cytotoxicity (6, 7). Several studies have shown that the effectiveness of different cell types may vary according to the nature of the target cell (for reviews, see 6, 7, 10). For example, working with bovine systems, we previously showed that whereas both lymphocytes and macrophages could destroy antibody-coated chicken erythrocytes, only macrophages proved efficacious against antibody-coated herpesvirus-infected targets (13). Later we demonstrated that highly purified populations of polymorphonuclear leukocytes (PMN) could also act as effector cells against both erythrocyte and virus-infected target cells (18). Exactly how the various cell types interact and possibly even counteract in causing the destruction of virus-infected cells needs definition. In the present communication, we have sought to better define the respective roles that various cell types might play in ADCC by critically comparing the efficiency of highly purified cell populations at mediating ADCC against antibody-sensitized herpesvirus-infected and erythrocyte target cells.

MATERIALS AND METHODS

Preparation of effector cells. Four populations of bovine cells were used for the investigation. The preparation of PMN from the mammary gland was previously described in detail (19). Briefly, cells were collected 6 to 10 h after the infusion of 5 μg of lipopolysaccharide (LPS) and were applied to Ficoll-Hypaque gradients. The PMN, which were present in the pellet, were washed three times with Hanks balanced salt solution and resuspended in Eagle minimum essential medium containing 10% fetal calf serum and buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (MEM-HEPES).

Macrophage preparation has also been described before (19). In brief, cells were obtained from the mammary gland 4 days after LPS stimulation. After Ficoll-Hypaque flotation, interface cells were washed twice in Hanks balanced salt solution, resuspended to 4×10^6 cell/ml in MEM-HEPES, and cultured for 2 to 3 h in plastic petri dishes. The nonadherent cells were removed by repeated washing, and the adherent cells (referred to as macrophages) were removed mechanically. These cells were washed and suspended to the desired cell concentration in MEM-HEPES.

Peripheral blood leukocytes were isolated by Ficoll-Hypaque flotation as described before (11). The interface cells were depleted of PMN and macrophages by passage through glass wool columns equilibrated with MEM containing 10% heat-inacti-

vated autologous plasma. These cells constituted the peripheral blood lymphocytes (PBL).

Granulocytes (PBG) were isolated from the pellet of the Hypaque gradients. The pelleted cells, which contained many erythrocytes, were resuspended in a large volume of 0.83% NH_4Cl for 5 min at 37°C to cause erythrocyte lysis. The cells were subsequently washed three times in HBSS and resuspended in MEM-HEPES.

Target cells. Details of Na_2 $^{51}CrO_4$ labeling of erythrocyte target cells and virus-infected targets were recently described in detail (13). Two systems were used: chicken erythrocytes (CRBC) and infectious bovine rhinotracheitis virus-infected Georgia bovine kidney cells (IBR-GBK). In most experiments, noninfected GBK cells were included as one of the controls.

ADCC assay. The methods used were exactly as described by Rouse et al. (13). The antisera used for sensitizing the CRBC and IBR-GBK target cells were bovine anti-CRBC and bovine anti-IBR sera, respectively. Assays were carried out in microtiter plates and included the following controls: target cells in MEM-HEPES, target cells in MEM-HEPES with sensitizing antisera, and target cells plus effector cells without sensitizing antiserum. At the concentrations used, the sera used were not toxic and did not increase the level of release of ^{51}Cr from target cells above the background release from target cells in MEM-HEPES alone. Results were expressed as the percentage of specific release (SR) and were computed by the following general formula:

$$SR = 100 \times \frac{\text{mean counts in test} - \text{mean counts in control}}{\text{total releasible counts} - \text{mean counts in control}}$$

Total releasible counts were obtained by suspending target cells in 1% Triton X-100.

RESULTS

Four cell populations, prepared from a single animal, were investigated for their ability to mediate ADCC against two target cell systems: (i) IBR-GBK sensitized with bovine anti-IBR serum and (ii) chicken erythrocytes sensitized with bovine anti-CRBC serum. The average composition of the four cell populations is shown in Table 1. The PBL, mammary PMN, and mammary macrophages consisted almost exclusively of one cell type, but the PBG were heterogeneous, with neutrophils forming the major type of cell.

In ADCC, PMN were invariably the most active effector cells tested (Fig. 1 and 2). This was true against both target cell systems and occurred at all effector cell/target cell ratios and at all concentrations of sensitizing antiserum investigated. Whereas PBL could act as effectors against CRBC targets, these cells had no activity against IBR-GBK targets. In the IBR-GBK system, PMN were often twice as active as macrophages. The activity of PBG was invariably less than 50% that of PMN and always less than that of purified macrophages. This result was surprising since PBG consisted of around 86% neutrophils. Of the contaminating cell types, both macrophages and lymphocytes do mediate ADCC against CRBC, but whether bovine eosinophils, like their murine counterparts (15), mediate ADCC has not yet been determined. One explanation for the lower lytic activity of PBG could have been that the NH_4Cl lysis step used during their preparation was toxic. This effect was reported by Yust et al. (20) with human cells. However, as shown in Table 2, NH_4Cl was toxic to neither mammary PMN, macrophages, nor PBL. Also included in Table 2 are results showing that the lytic activity of PBL against CRBC increased markedly upon culture. However, this increase could not be attributed to a recovery from NH_4Cl toxicity, since these cultured cells were not inhibited by a subsequent treatment with NH_4Cl. Cultured

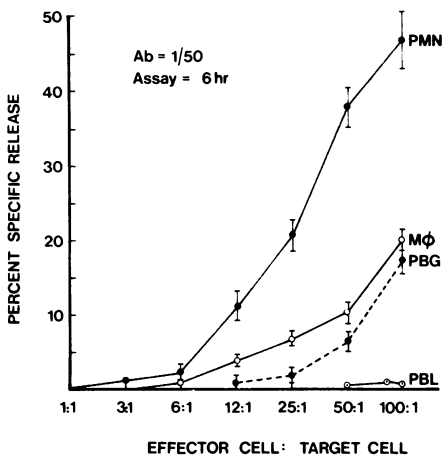
TABLE 1. *Average composition of cell preparations as determined by histological examination of Wright-Giemsa-stained smears*

Cell prepn	Neutrophils (%)	Macrophages (%)	Lymphocytes (%)	Eosinophils (%)
PMN[a]	99 (98–100)[b]	0.75 (0–1)	0.25 (0–1)	0
Macrophages[a]	0.5 (0.5–1)	97 (95–98)	2.5 (2–4)	0
PBG[c]	86 (84–90)	1 (0.5–2)	4 (2–6)	9 (8–12)
PBL[c]	0	4	96 (95–99)	0.3

[a] Cells collected from the mammary gland. The PMN were collected 6 to 10 h after stimulation of the gland with 5 μg of LPS. The cells were further purified by Ficoll-Hypaque flotation where they appeared in the pellet. Macrophages were prepared from cells collected 4 days after LPS stimulation. The cells used were collected from the interface of Ficoll-Hypaque gradients and then adhered to plastic for 2 to 3 h. The adherent cells were removed by mechanical means.

[b] Figures in parentheses represent range values from five different preparations.

[c] Cells prepared from peripheral blood. Buffy-coat cells were centrifuged on Ficoll-Hypaque gradients, and the interface cells were passed over glass wool columns. The nonadherent cells consisted of 38 to 40% cells with surface immunoglobulin. Between 30 and 35% of the cells contain Fc and complement receptors (manuscript in preparation).



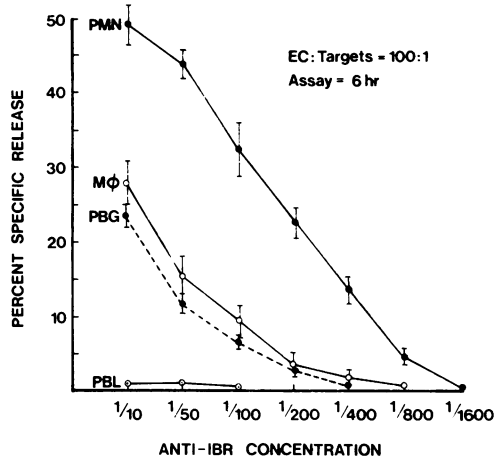


FIG. 1. *Comparison of ADCC activity of mammary PMN, mammary macrophages (Mφ), PBG, and PBL against herpesvirus-infected bovine target cells sensitized with bovine anti-IBR serum. (Left) Influence of effector cell/target cell ratio at a constant antibody concentration. (Right) Effect of varying antibody concentrations. Background release, 15.8%. Specific cell lysis did not occur in the absence of anti-IBR serum.*

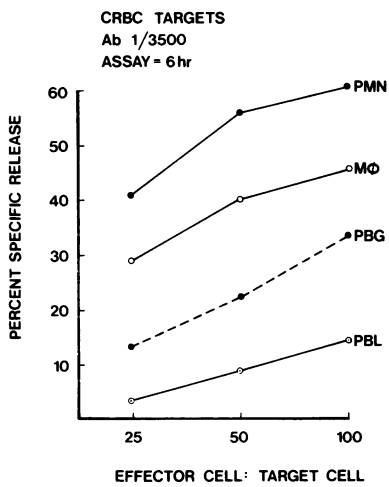


FIG. 2. *Comparison of ADCC activity of mammary PMN, mammary macrophages (Mφ), PBG, and PBL against chicken erythrocytes sensitized with bovine anti-CRBC serum. Background release, <1%. Specific cell lysis did not occur in the absence of anti-CRBC sera.*

PBL were still without lytic activity against IBR-GBK targets.

In experiments designed to study the kinetics of target cell lysis, PMN were shown to be the most active cell type at all time intervals (Fig. 3 and 4, Table 3). Thus lysis of IBR-GBK targets was first detectable at 60 to 90 min, around 60 min earlier than macrophage- or PBG-mediated lysis. However, with all cell types, the times of peak lysis were identical. A comparison of the lytic activity of PMN and macrophages at various times against IBR-GBK targets sensitized with different concentrations of antiviral antibody is shown in Table 3. At a high antibody concentration, lysis occurred earlier with both effector cells: at a 1/20 antibody concentration, PMN-mediated lysis occurred 1 h before macrophage-mediated ADCC, but at a 1/1,000 antibody concentration, the time differential of lysis detection was 2 h. The results also show that the relative ADCC effectiveness of PMN and macrophages varied according to the time of assay. Thus the ratio of PMN to macrophage activity diminished with increased incubation time.

DISCUSSION

In support of previous studies with bovine cell types, we have shown that ADCC against herpesvirus-infected target cells can be mediated by macrophages and PMN but not by lymphocytes (13). On the other hand, chicken erythrocytes are destroyed by cells of all three above-mentioned types. The present communication has extended previous work by critically comparing the effectiveness of highly enriched cell populations, taken from the same animal, in mediating ADCC. By several approaches, PMN, obtained from the mammary gland, were the most active in ADCC against both target cell systems. Thus against virus targets, lysis was detected earlier, reached higher levels, and occurred at lower effector cell/target cell ratios with PMN than macrophages. Furthermore, PMN-mediated ADCC was detectable at lower levels of sensitizing antiserum than was needed by macrophages. The pattern of results with

TABLE 2. *Effect of NH_4Cl treatment on the efficacy of different effector cell types in mediating ADCC*

Cell type[a]	Culture time[b] (h)	Treatment[c]	CRBC[d]	IBR-GBK[e]
PMN	0	None	43.5 ± 2.5[f]	40.5 ± 0.5[f]
PMN	0	NH_4Cl	40.0 ± 3.5	39.0 ± 1.0
Macrophages	0	None	31.0 ± 1.0	33.5 ± 1.5
Macrophages	0	NH_4Cl	32.0 ± 2.0	32.0 ± 1.2
PBL	0	None	7.0 ± 1.0	0
PBL	24	None	44.0 ± 4.0	0
PBL	24	NH_4Cl	44.0 ± 2.0	0

[a] All cells were used at an effector cell/target cell ratio of 100:1.

[b] Cells were held in culture at 37°C in MEM containing 10% fetal calf serum before being used in the ADCC assay.

[c] Cells were held at 5×10^6/ml in 0.83% NH_4Cl for 5 min at 37°C. Cells were then washed twice, suspended to the desired concentration in MEM-HEPES, and then tested immediately for ADCC.

[d] ^{51}Cr-labeled chicken erythrocytes sensitized with a 1/3,500 concentration of bovine anti-CRBC serum. Assay was performed for 6 h. Background release was less than 1%. Specific cell lysis did not occur in the absence of anti-CRBC serum.

[e] ^{51}Cr-labeled GBK cells were infected with 1 plaque-forming unit of IBR virus per cell 16 h before the start of assays. Bovine anti-IBR serum was used at a final concentration of 1/50. Assay time was 8 h; background release was 12%. Specific cell lysis did not occur in the absence of anti-IBR serum.

[f] Average specific release ± standard error.

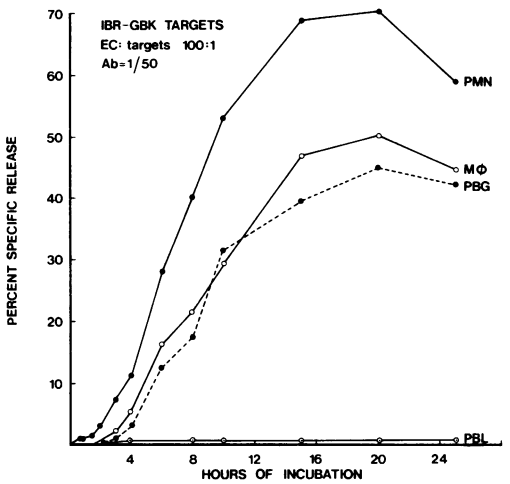


FIG. 3. *Kinetics of lysis of herpesvirus-infected target cells by mammary PMN, mammary macrophages (MΦ), PBG, and PBL. Background release ranged from 12.1% at 4 h to 38% at 24 h. Specific cell lysis did not occur in the absence of anti-IBR sera.*

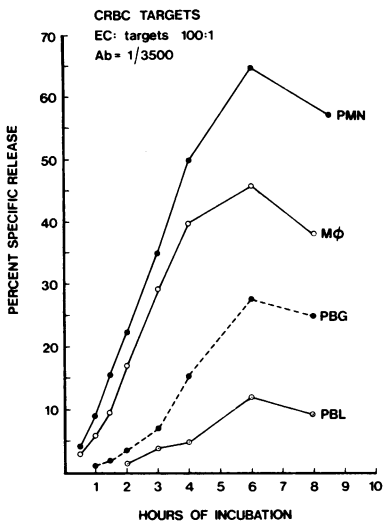


FIG. 4. *Kinetics of lysis of chicken erythrocyte target cells by mammary PMN, mammary macrophages (MΦ), PBG, or PBL. Background release was 1.2% at 9 h. Specific cell lysis did not occur in the absence of anti-CRBC serum.*

CRBC targets was similar: of the three enriched cell types that mediated ADCC, the order of effectiveness was PMN > macrophages > PBL. Others have shown previously that murine PMN were more active than other cell types at mediating ADCC against erythrocyte targets, but against tumor targets the same cells were without activity (5). However, tumor cells can be destroyed by human PMN (3), but whether PMN were superior to other cell types in mediating ADCC was not reported.

Why any one cell type is effective in ADCC and why the respective efficiency of different cells varies according to the target system are unresolved questions. It seems that binding by means of an Fc receptor is a necessary (6) but perhaps not sufficient step for lysis to occur (20). Whether the differences in lytic activity by different cells can be accounted for by differences in Fc receptor number, specificity, or binding avidity or whether membrane proper-

TABLE 3. *Kinetics of lysis of herpesvirus-infected GBK cells sensitized by different concentrations of antiviral serum by PMN and macrophages*

Time (h)	%SR[a]								
	1/20[b]			1/200			1/1,000		
	PMN	Mϕ[c]	PMN/Mϕ	PMN	Mϕ	PMN/Mϕ	PMN	Mϕ	PMN/Mϕ
0.5	0	0		0	0		0	0	
1	3.3	0	∞	0	0		0	0	
2	14.2	1.0	14.2	0	0				
3	32.2	3.6	8.8	5.3	0	∞	1.2	0	∞
4	40.8	7.1	5.7	12.0	3.0	4.0	2.3	0	∞
6	57.8	29.1	2.0	21.7	8.2	2.6	10.0	3.3	3.0
10	59.4	53.4	1.1	38.9	31.1	1.2	29.1	17.7	1.6
18	67.4	60.6	1.1	52.7	33.8		37.5	20.2	1.9

[a] Mean percentage of specific release (SR) from four replicates.
[b] Concentration of bovine anti-IBR serum. Specific lysis did not occur in the absence of anti-IBR serum.
[c] Mϕ, macrophages.

ties of the target cells account for the differential effects must be resolved. In the present study, we have shown that a given effector cell type isolated from different regions of the same animal may apparently differ in its ability to mediate ADCC. Thus PBG (86% PMN) were less than 50% as effective as mammary PMN (99% PMN) in mediating ADCC. Several explanations for these results can be given, including the possibility that only certain subsets of PMN mediate ADCC, with the relative proportion of subsets varying according to the source of cells; cell types other than PMN present in the PBG inhibit the activity of PMN; or the activity of PMN is inhibited by the cell preparation methods used. Regarding the latter explanation, others have shown that NH_4Cl, used to lyse erythrocytes, can cause a temporary inhibition of ADCC activity (20). However, in our system, NH_4Cl failed to suppress the activity of mammary PMN or macrophages, making this an unlikely explanation for the poorer lytic activity of PBG.

The mammary PMN used were induced in response to LPS infusion and consequently represented acute inflammatory cells. Others have shown that LPS can change the activity of macrophages (2) and that stimulated macrophages may show changes in Fc receptor function (9). It must be shown whether PMN react in a similar way to macrophages and whether such activated cells react differently in ADCC.

At present we have not evaluated the possibility that one of the minor cell types in the PBG population was inhibiting the activity of PMN. The feasibility of this explanation is supported by the work of Sanderson and Taylor, who showed, using murine effector cells against antibody-sensitized erythrocyte targets, that macrophages inhibited lymphocyte-mediated lysis (14). In our system macrophages probably would not be the candidate cells for inhibition, because whereas in Sanderson and Taylor's study macrophages did not mediate ADCC, bovine macrophages certainly can do so. We are trying to evaluate the possibility that other cell types (eosinophils and lymphocytes) in the PBG preparation have inhibitory effects on PMN-mediated ADCC.

If ADCC has any relevance at all to an in vitro defense mechanism, then perhaps the most important aspect of our study is the implication that PMN can play a major role in recovery from herpesvirus infections. Thus the facts that PMN were more effective on a cell-to-cell basis, required less antiserum to mediate ADCC, and destroyed virus-infected cells faster and more completely than other cells types could mean that PMN are crucially important, especially early in the recovery process, when levels of antiviral antibody are low. We are currently designing in vitro models to assess the respective importance of PMN and their interplay with other cell types in causing recovery from herpesvirus infections.

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