Brief Definitive Report

ANALYSIS BY A PLAQUE ASSAY OF IgG- OR IgM-DEPENDENT CYTOLYTIC LYMPHOCYTES IN HUMAN BLOOD*

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Whereas the capacity of IgG antibodies to induce a cytolytic activity in certain lymphocytes (K cells) is well established (1), that of IgM antibodies is considered to be of doubtful significance (2, 3). In fact, it has been shown that IgM antibodies may inhibit the IgG-dependent reaction (4). However, Lamon et al. (5, 6) have now provided evidence that IgM antibodies may induce cytolysis of murine sarcoma virus-induced mouse tumor cells by mouse lymphocytes, including T cells. It has also recently been reported that a significant proportion of human peripheral T lymphocytes form rosettes with IgM-coated erythrocytes by means of receptors distinct from the cellular Fc receptors for IgG (7, 8). We therefore reinvestigated the question of the relative capacities of IgG and IgM to induce lymphocyte-mediated target cell lysis in man. We made use of a plaque assay in which K-cell activity is determined on the level of the individual effector cells (9, 10).

Materials and Methods

Highly purified lymphocytes from defibrinated human blood were used (11). The lymphocytes were used either untreated or treated with neuraminidase (NANAase, from *Clostridium perfringens*) (12). Target cells in the cytolytic reactions were bovine erythrocytes (E_b). Cell-mediated erythrolysis was induced with either the IgG or the IgM fraction of a rabbit anti-E_b serum (13). The protein fractions were obtained by gel exclusion on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). The concentration of specific anti-E_b antibodies was determined by measuring binding of ^{125}I-labeled protein to E_b. Different dilutions of trace-labeled IgG or IgM were added to a constant number of E_b in excess. ^{125}I-labeled IgG or IgM fraction from a normal rabbit serum, previously absorbed with E_b, were used to determine the background that was subtracted. The antibody concentration was 9.5% of the protein in the IgG fraction and 7% in the IgM fraction.

Cell-mediated erythrolysis on the single cell level was determined as described (13). In brief, 0.5 ml of 1% E_b ($\sim 10^8$ cells) was added to poly-L-lysine-treated coverslips in plastic Petri dishes (35 × 10 mm). They were incubated for 45 min and washed to form dense E_b monolayers. To these were added 0.5-ml dilutions of the inducing rabbit antibodies and 0.5 ml lymphocytes (0.5–4 × 10^6/ml, corresponding to lymphocyte/E_b ratios of 1:20–1:80 per mm^2 on the coverslips). The medium was supplemented tissue culture medium RPMI 1640 (Biocult, Paisley, Scotland) and contained 10% heat-inactivated fetal bovine serum (FBS) (Flow Co., Irvine, Scotland). After gentle agitation the dishes were incubated at 37°C in air plus 5% CO_2. Lymphocyte-free controls and controls containing lymphocytes and normal rabbit serum fractions or FBS but no antiserum were set up in

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parallel. In the inhibition experiments, purified human IgG or IgM proteins were added to the plates before addition of the lymphocytes. IgG was isolated from normal serum by DEAE-cellulose chromatography (14). The IgM was a cryoglobulin from a patient with Waldenström's macroglobulinemia. It was purified by four precipitations in an ice bath, followed by passage at 37°C over Sepharose-coupled protein A from *Staphylococcus aureus* (Pharmacia Fine Chemicals Inc.).

For enumeration of the number of plaque-forming cells (PFC), the monolayers are briefly fixed with dilute glutardialdehyde (GA) (13). E_b lysis is reflected by the formation of clear zones of irregular shapes and different sizes. An area corresponding to ≥ 5 lysed E_b is defined as a plaque. In general, 1 or rarely 2 or more lymphocytes are seen associated with ~80% of the plaques. The number of PFC as percentage of the total number of lymphocytes added is calculated as $100 \times a \times b/c \times d$, where a denotes total area of dish, b denotes number of plaques counted in a phase contrast microscope, c denotes number of lymphocytes added, and d denotes total area screened for counting plaques in randomly chosen fields. This estimate is based on the assumption that one K cell does not form more than one plaque. However, since some of the larger plaques may be formed by confluency and since some of the minor plaques may escape detection, these estimates give minimal numbers and the actual number of PFC may be larger (10, 13).

Surface markers were determined in situ on lymphocytes either associated with plaques or found on the intact E_b monolayers (13). Lymphocytes with complement receptors were assayed by binding of EAC (E, sheep erythrocytes; A, IgM fraction of rabbit anti-sheep erythrocyte serum; C, C6-deficient rabbit serum) to the lymphocytes on unfixed coverslips and fixation with 1% GA after incubation for 60 min at 37°C. Surface bound immunoglobulin (SIg) was determined in situ by direct immunofluorescence with FITC-labeled rabbit anti-human $F(ab')_2$ reagent, added after 20-s fixation of the plates with 0.5–1% GA. Receptors for *Helix pomatia* A hemagglutinin (HP) were assayed on lymphocytes that had been treated with NANAase in Tris-buffered Hanks' solution containing 0.2% human serum albumin before addition to the monolayers (12). Staining of the plates with TRITC-labeled HP protein was performed as described for SIg staining. Double marker staining (HP-SIg) with TRITC-labeled HP protein followed by FITC-labeled anti-immunoglobulin was performed according to Hellström et al. (15).

Results and Discussion

The time-course of the IgM-dependent plaque formation by either untreated or NANAase-treated lymphocytes is shown in Fig. 1. As in the IgG-dependent reaction NANAase-treated lymphocytes are significantly more active than untreated lymphocytes. However, while the IgG-dependent reaction under the present conditions proceeds linearly to completion at about 20 h of incubation (16), the IgM-dependent reaction is slower but proceeds for approximately 40 h before it stops. These results are compatible with those of the rosette tests which indicate that IgM receptors on lymphocytes require prolonged incubation at 37°C to become fully expressed (7, 8). While the minimal number of PFC for IgG at optimal concentration in nine different donors was $5.6 \pm 1.2\%$ of the lymphocytes added (mean $\pm$ SE), it was $2.0 \pm 0.8\%$ for IgM, in spite of the fact that the molar concentration of IgM antibodies used in these experiments was $2 \times$ higher than that of the IgG antibodies. The mean size of the IgM plaques was smaller (~5–10 lysed E_b/plaque) than that of the IgG-plaques (~25 lysed E_b/plaque). Taken together, these results would seem to suggest that the effector cells are a small subset of the lymphocytes which form rosettes with IgM-coated E_b (7, 8, 17), and that the IgM-dependent cytolytic system is less efficient than the IgG dependent one. However, these questions need to be explored further. Thus, the optimal reactions in the plaque test are obtained at low lymphocyte/E_b ratios (13, 16), and the lowest number of lymphocytes giving the maximal relative frequency of PFC has not as yet been determined. The optimal concentration of the IgM antibody also remains to be established.

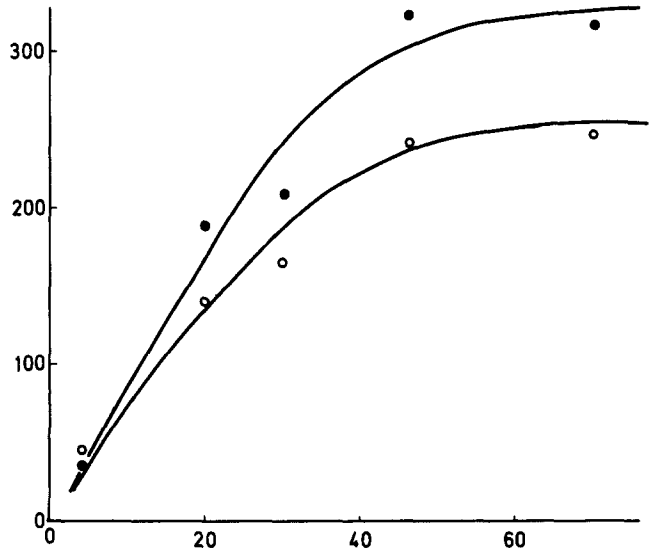

FIG. 1. Time-course of plaque formation. E_b monolayers treated with 10.5 μg rabbit IgM antibodies and 2 × 10^6 lymphocytes/dish (total vol 1.0 ml). Lymphocytes either untreated (circles) or treated with NANAase before addition to the dishes (dots). Ordinate: total number of plaques counted, corrected by subtracting plaques found in control (≤4). Abscissa: hour of incubation.

Fig. 2 shows the results of a typical inhibition experiment, with human immunoglobulins used as inhibitors. The lymphocytes were from the same donor. The results indicate that the immunoglobulin receptors on the effector cells in these two cytolytic systems are different. This is similar to what has been noted for rosette formation (7, 8, 17).

Very similar results have been obtained in experiments in which the IgM-induced cellular cytotoxicity was established by measuring ^{51}Cr release from labeled E_b in suspension (unpublished). Several reasons may account for previous negative results with IgM antibodies (2–4). One possible explanation of the present positive results may be the relative inagglutinability of E_b (18) which facilitates their coating with the critical amounts of IgM required for cytotoxicity. The present results do not contradict previous findings of an inhibitory activity of IgM antibodies on the IgG-dependent reaction (4). This inhibition reflects a competition for antigenic sites on the target cells and obviously depends on the quantitative balance of the different reactants. Inhibition should become particularly important if the IgG- and IgM-dependent effector cells were different lymphocytes. That this may be the case to a significant extent is suggested by surface marker analysis. Typical results of an experiment performed with NANAase-treated lymphocytes are given in Table I. The distribution on the original lymphocytes of the three surface markers was the same as that earlier described (12). The surface marker profile of the lymphocytes seen on the intact monolayers was similar to that of the original preparation, thus suggesting a random distribution of the lymphocytes in these areas. In contrast, the plaque-associated cells had different profiles, in accord with the notion that

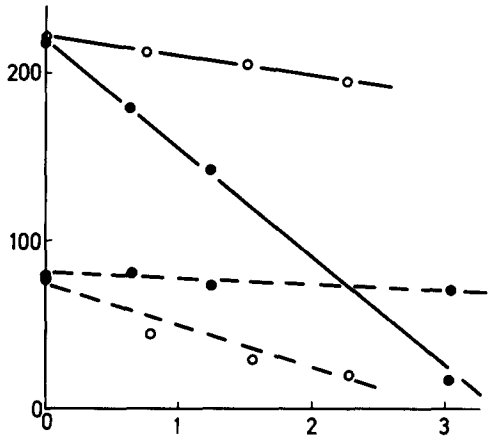

FIG. 2. Inhibition of IgG-dependent (solid lines) or IgM-dependent (broken lines) plaque formation by human IgG (dots) or IgM (circles). Ordinate: total number of plaques counted. Abscissa: milligrams inhibitor per dish (total vol 1.2 ml). For other details see Fig. 1 and text.

TABLE I
Surface Marker Profiles

Lymphocytes*	Percent cells with surface marker					
	EAC+	SIg+	HP+	Double staining		
				SIg+-HP+	SIg+-HP-	SIg--HP+
Original suspension	23	10	75			
IgG-dependent reaction§						
Plaque associated (PFC)	47	11	27	12	0	21
On monolayer	21	13	66	7	5	62
IgM-dependent reaction‡						
Plaque associated (PFC)	7	11	58	12	0	43
On monolayer	ND	12	69	5	3	66

ND, not done

* Lymphocytes treated with NANAase before incubation on E_b monolayers (12, 13)

§ E_b monolayers treated with 1.5 μg rabbit IgG antibody and 2×10^6 lymphocytes/dish, total vol 1 0 ml Time of incubation. 20 h, minimal number of PFC 5.9 % of lymphocytes added

‡ E_b monolayers treated with 10.5 μg rabbit IgM antibody and 2×10^6 lymphocytes/dish Time of incubation: 40 h, minimal number of PFC 1.8%

the majority of the plaque-associated cells represent PFC, i.e., K cells (10, 13). As previously shown, ~50% of these cells in the IgG system had complement receptors (EAC^+) and ~30% were HP^+, while the percentage SIg^+ cells was similar to that seen in the original preparation and on the intact monolayer. Double staining of these cells for SIg and HP receptors (15) indicated that all plaque-associated SIg^+ cells also were HP^+. Thus, these cells belong to the subset of the HP^+ lymphocytes (10–20% in normal blood) which also have Fc receptors for IgG and externally adsorbed surface IgG (12). It will be noted that the SIg-HP profile of the lymphocytes studied by double labeling on the intact monolayer was different.

Table I further shows that the surface marker profile of the plaque-associated

cells in the IgM-dependent reaction was different in that only few cells had complement receptors while ~60% were HP^+. Moreover, ~80% of the latter were SIg^-. Therefore a large fraction of the IgM-dependent K cells are probably T cells which have previously been shown to be HP^+-SIg^--EAC^- (12). These cells are distinct from the majority of the IgG-dependent effector cells. However, it can not be excluded that they also have Fc receptors for IgG and IgG-dependent cytolytic potential. The degree of overlap between different effector cell types as well as the cellular origin of the immunoglobulin receptors involved requires further studies.

In conclusion, we have shown that IgM may induce a cytolytic activity in human T cells as it does in the mouse (5, 6). The biological role of this new effector mechanism remains to be established. Since these antibody-dependent killer cells are a subset of the peripheral T-cell population, it will be of great interest to elucidate their relationship to other T-cell subsets, in particular to the antibody-independent T-killer cells (CTL) (1).

Summary

When monolayers of bovine erythrocytes (E_b) were exposed to purified human blood lymphocytes and either IgG or IgM fractions of rabbit anti-E_b serum, clear zones (plaques) appeared where E_b had been lysed by antibody-dependent effector cells (K cells). IgG-dependent plaque formation was complete by 20 h of incubation, while the IgM-dependent reaction required 40 h. The estimated minimal numbers of plaque forming cells (PFC) were 5.6% (IgG) and 2.0% (IgM) of the added lymphocytes. Inhibition experiments with human IgG or IgM indicated that different immunoglobulin receptors on the effector cells were involved in the two systems. In the IgG system, ~50% of the PFC had complement receptors and ~30% receptors for *Helix pomatia* A hemagglutinin (HP). In the IgM system, <10% of the PFC had complement receptors, while ~60% had HP receptors. The results suggest that a subset of human T cells have IgM-dependent K-cell potential. These cells are different from the majority of the IgG-dependent K cells.

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