Fc receptor triggering induces expression of surface activation antigens and release of platelet-activating factor in large granular lymphocytes

(immunoglobulin receptor/DR/DQ antigens)

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ABSTRACT  Triggering of large granular lymphocyte (LGL) Fc receptor with a specific monoclonal antibody (AB8.28) linked to an insoluble matrix induces cell activation, as witnessed by expression of HLA class II (DR and DQ) molecules and interleukin 2 receptor. Moreover, this event is accompanied by a concomitant release of platelet-activating factor by LGL. We conclude that the Fc receptor molecule identified by mAb AB8.28 represents a trigger for LGL activation.

Large granular lymphocytes (LGL) constitute a discrete population of peripheral blood mononuclear cells that perform several functions, among which natural killing is the best known. Recent evidence (1) suggests that only a part of the entire cellular potential of LGL is known and other novel regulatory and functional properties may be attributed to them. The study of LGL using monoclonal antibodies (mAbs) has made it possible to define a gross map of the cell surface that includes structures expressed by the majority of cells displaying LGL features and others, such as Leu7, C3b receptor, or N901 (2), that are present on variable proportions of the population. A general observation is that many markers present on LGL are also expressed by cells belonging to other lineages; furthermore, most of these markers are located on molecules whose functions are unknown. A relevant exception is the molecule recognized by mAbs AB8.28 (3), Leu11, B73.1, 3G8, and VEP13, which appear to identify an Fc receptor (FcR) for human IgG (4). These reagents also react with the FcR of polymorphonuclear neutrophils (PMN), but they do not recognize the FcR of B lymphocytes and monocytes (4), which supports the view that FcR are a family of multiple molecules. The aims of this study were 2-fold: (i) to investigate if the FcR identified by mAb AB8.28 could function as a molecule switch for cell activation and (ii) to identify the main events occurring in LGL after antibody triggering. We present evidence that the stimulation of LGL FcR with mAb AB8.28 covalently bound to an insoluble matrix (Sepharose CL-4B) induces LGL activation and expression of HLA class II (DR and DQ) and interleukin 2 (IL-2) receptor (TAC) antigens. Simultaneously, we observed that large granulocyte platelet-activating factor (PAF), a powerful mediator of cell–cell interaction (5) with an in vitro toxic effect on cultured normal and neoplastic cells (6). These data may provide a clue toward understanding the initial steps in activation of the natural killer (NK) cell activity in vivo.

MATERIALS AND METHODS

mAbs. mAb AB8.28 was obtained with peripheral blood lymphocytes (PBL) of a healthy blood donor as the immunogen (3). Other mAbs used for phenotypic studies were R1.30 (anti-B2-microglobulin), O1.65 (anti-HLA class I), A10 and BT3/4 (anti-HLA DQ1) (Technogenetics, S. Mauro T., Italy), AA3.84 (anti-HLA DR) (7), Tu22 (anti-HLA DQ) (6), Leu1, Leu7, Leu11, and anti-T-TAC (Becton Dickinson), CB01 (anti-T cells and thymocytes) (unpublished), CB12 (anti-monocytes) (9), and Mo1 and B1 (Coulter).

Preparation of Cells. PBL of healthy donors were isolated from heparinized blood by density-gradient centrifugation using Ficoll/Hypaque (Pharmacia), depleted of plastic-adherent monocytes, and resuspended in RPMI 1640 medium. The percentage of macrophages/monocytes never exceeded 2% (10). T cells, PMN, and monocytes were prepared as described (11). In a limited number of experiments, an AB8.28+ subset was purified by negative selection by depletion with immunorosette and anti-pan T, anti-monocytes, anti-HLA class II, and anti-B1 along with removal of plastic-adherent cells.

Immunorosetting. Ox erythrocytes were coupled to protein A in the presence of 0.01% CrCl3 using the procedure of Ling et al. (12). After sensitization with mAb, PBL were mixed with a 1% ox erythrocyte-protein A suspension and centrifuged for 5 min at 160 × g. Rosette-forming cells were separated by Ficoll/Hypaque density-gradient centrifugation and recovered after osmotic lysis of the protein A-coupled ox erythrocytes. To eliminate any residual mAb AB8.28 on the surface, the sorted cells were allowed to immunomodulate the antibody–target molecule complex by incubating for 12 hr at 37°C with several changes of RPMI 1640 medium supplemented with 10% human AB serum.

Labeling of the mAb AB8.28 and Immunofluorescence Assay. AB8.28 was purified from ascites fluid and labeled with biotin (Enzo Biochemicals, New York) (3). The biotinated mAb was revealed by fluorescein isothiocyanate- or phycoerythrin-conjugated avidin (Becton Dickinson). Indirect immunofluorescence was performed with a Leitz inverted microscope equipped with epifluorescence, by allowing 1 × 10⁶ cells to react with appropriate dilutions of mAbs (8). In selected experiments, FITC-labeled mAbs were used in direct tests.

Conjugation of mAb AB8.28 to Sepharose. Purified AB8.28 was covalently linked to activated Sepharose CL-4B

Abbreviations: LGL, large granular lymphocyte(s); mAb, monoclonal antibody; PMN, polymorphonuclear neutrophil(s); FcR, Fc receptor(α); PAF, platelet-activating factor; NK, natural killer; PBL, peripheral blood lymphocyte(s); Ac-CoA, acetyl-coenzyme A; IL-2, interleukin 2.
(Pharmacia) following the cyanogen bromide technique (13) (10 mg of mAb per 1 g of dry beads).

**Fcr Triggering.** AB8.28+ cells \((2 \times 10^6)\) were incubated with 50 µl of AB8.28–Sepharose overnight at 37°C in a humidified atmosphere with 5% CO2 in air. The choice of 50 µl of dry pellet of mAb–Sepharose was made on the basis of several experiments and represents an excess amount. The culture spent medium was recovered, the Sepharose was removed by Ficoll/Hyaque density-gradient centrifugation, and the cells were tested with the selected mAbs.

**PAF Release and Assay.** AB8.28+ cells were washed in Tris/Tyrode buffer/bovine serum albumin (14) and incubated with calcium ionophore A23187 (Boehringer Mannheim, 2 µg/ml) and 50, 10, and 1 µl of AB8.28–Sepharose, O1.65–Sepharose, and AA3.84–Sepharose, respectively. In some experiments, acetyl-coenzyme A (Ac-CoA, 0.2 mM) was added to the cells. The cell-free supernatants were extracted in methanol, and the lipid material was phased into chloroform (15) and purified as described below. As controls, 1 \( \times 10^6 \) PMN and 1 \( \times 10^6 \) monocytes were challenged with A23187 (2 µg/ml) and AB8.28–Sepharose (50, 10, 1 µl). The cell supernatants were then processed as above. PAF was detected by aggregation of rabbit platelets \((2-5 \times 10^8)\) in Tris/Tyrode buffer containing 0.25% gelatin in the presence of indomethacin and the creatine phosphate/creatine phosphokinase enzymatic system. The amount of PAF was quantitated and expressed as described (14).

**Purification and Characterization of PAF.** PAF was purified by TLC (15). The lipid material with a retention front \((R_f = 0.21)\) identical to that of synthetic 1-O-octadecyl-2-acetylsn-glycero-3-phosphocholine (Bachem, Bubendorf, Switzerland) (PAF) was further characterized by its retention time on a HPLC apparatus (Beckman). Elution was carried out on a linear gradient over 15 min (16) and the fractions were bioassayed on rabbit platelets. Identity between HPLC-eluted material and synthetic 1-O-octadecyl-2-acetylsn-glycero-3-phosphocholine was established on the basis of similar physical and chemical characteristics and sensitivity to lipases (17).

**RESULTS**

**Cell Preparation.** The AB8.28+ subset was isolated with immunorosettes from PBL preparations after stepwise depletion of plastic adherent and sheep erythrocyte-rosetting cells. The resulting population was >90% A10+ and >95% AB8.28+ and Leu11+ with the morphologic features of LGL and cytotoxic activity (data not shown) (3). The majority of these cells weakly expressed Mo1, whereas only 30–60% of them reacted with Leu7. The molecules detectable with the anti-pan T, anti-monocytes, and anti-DR mAbs were present in <1% of the AB8.28 subsets. The capping occasionally observed in a limited number of cells was eliminated by extending the incubation by 2 hr at the same conditions described above. After immunomodulation, the cells were completely free of surface-bound mAb, and their phenotype did not differ significantly from unmodulated cells.

**Fcr Triggering and Expression of Activation Markers.** Purified LGL preparations were challenged with anti-FcR mAb with the antibody in either soluble or immobilized form. As controls, we used anti-HLA class I (positive) and class II (negative) antibodies under the same conditions. After Fcr triggering, >95% of LGL expressed AB8.28 and Leu11, whereas 30–60% expressed Leu7; a variable number of cells (ranging from 45% to 70% in different experiments) expressed human HLA.A. To investigate the possibility of differential expression of HLA class II products, we used directly fluoresceinated or biotinated reagents specific for DR (AA3.84) and DQ (Tu22) determinants. The polymorphic anti-DQ1 reagent BT14/3 was used in selected patients who had been typed previously as HLA DQ1. Double-staining experiments demonstrated that nearly all of the cells coexpressed DR and DQ determinants (data not shown). Similar results were obtained with anti-TAC antibody: ~15–55% of the cells became strongly TAC+ after AB8.28 stimulation, in contrast to the resting AB8.28+ cells, which did not express this antigen (Table 1). The A10 molecule, which is detectable on the majority of resting LGL, showed a marked increase in the intensity of expression. No activation was observed among the controls. Finally, the same results were obtained with the AB8.28+ cells after removal of any contaminating T cells or monocytes by specific immunorosetting or after negative selection of the AB8.28+ subset, which, by the way, is reported to induce complete abolition of the chemiluminescent response (18).

**PAF Release.** Treatment with immobilized anti-FcR was able to induce NK cells to release PAF. No effect was observed when cells were treated with either soluble or

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> **Table 1.** Cell-surface phenotype and immunofluorescence score of stimulated LGL.

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<th>AA3.84</th>
<th>Tu22/BT3/4</th>
<th>TAC</th>
<th>A10</th>
<th>Leu1/CB01</th>
<th>CB12</th>
<th>X63.Ag8</th>
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<tr>
<td>AB8.28–Sepharose</td>
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<td>++</td>
<td>+++</td>
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<td></td>
<td>(35–70%)</td>
<td>(30–65%)</td>
<td>(15–50%)</td>
<td>(&gt;90%)</td>
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<td>AB8.28 mAb</td>
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<td>O1.65–Sepharose</td>
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<td>AA3.84–Sepharose</td>
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+++ , Very strong ring-like pattern; +++, clearly defined ring-like pattern; +, weaker but clear.

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> **Table 2.** PAF release from AB8.28+ cells, monocytes, and PMN

<table>
<thead>
<tr>
<th></th>
<th>AB8.28–Sepharose, µl</th>
<th>O1.65–Sepharose, µl</th>
<th>AA3.84–Sepharose, µl</th>
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<tbody>
<tr>
<td>A23187, 2 µg/ml</td>
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<tr>
<td>AB8.28+ cells ((2 \times 10^6))</td>
<td>9.2 ± 2.3</td>
<td>7.3 ± 1.4</td>
<td>0</td>
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<tr>
<td>Monocytes ((1 \times 10^6))</td>
<td>20.0 ± 3.5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>PMN ((1 \times 10^6))</td>
<td>11.1 ± 2.1</td>
<td>2.1 ± 3.2</td>
<td>0</td>
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Results are expressed in ng of PAF per ml (mean ± SD) (six experiments). ND = not determined.
immobilized anti-HLA reagents or with purified mAb AB8.28, even in excess amounts (10–1000 μg per 2 × 10⁶ cells). The ability of LGL to release PAF was confirmed afterward by treating the cells with A23187, a PAF-releasing agent. The same effect was observed with monocyte and PMN control populations (Table 2). To test the specificity of the effect observed, the same preparations were challenged with insolubilized AB8.28 mAb. PMN could also be triggered (though to a much lower extent), in contrast to monocytes, which were totally unresponsive. However, no PAF was released from LGL challenged with Sepharose beads coated with mAb O1.65 or AA3.84. The release of PAF from LGL was maximal 45 min after challenge with mAb AB8.28–Sepharose (Fig. 1A) and A23187 (Fig. 1B). In quantitative terms, PAF release obtained from 2 × 10⁶ LGL after challenge with A23187 was comparable to that obtained from 1 × 10⁶ PMN (Table 2). As shown in Fig. 2, at least 1 × 10⁶ LGL were necessary to observe detectable amounts of PAF after challenge with A23187 and AB8.28–Sepharose. Ac-CoA enhanced the release of PAF from LGL stimulated with A23187 and AB8.28–Sepharose, probably by acting as a substrate for PAF biosynthesis. The lipid material with a Rf of 0.21 on TLC was further purified by HPLC and shown to share with PAF (i) the retention time (Fig. 3), (ii) the activity on rabbit platelets, (iii) physical and chemical characteristics (16), and (iv) the inactivation by phospholipase A₂ (17).

DISCUSSION

Some features of the cytotoxic activity of NK cells, such as absence of HLA restriction and lack of memory, have been described previously: recent data indicate that cytoplasmic granules (from LGL or cytotoxic lymphocytes) play a key role in formation of membrane lesions on target cells [for review, see Podack (19)]. The phenotype of LGL is better known; the FcR found on LGL is different from the one expressed by human monocytes and B lymphocytes but appears to be similar to that reported on PMN. Functional data are limited to inhibition of EA7S rosette formation, IgG-mediated phagocytosis, and binding of immune complexes.

The aim of the present work has been to determine whether stimulation of the LGL FcR molecule can trigger activation of LGL and release of soluble factors. After using the approach that was successfully utilized in analyzing the T-cell receptor, the antibody anti-FcR covalently bound to an insoluble matrix (Sepharose CL-4B) was able to induce LGL to express surface “activation” markers and to concomitantly release PAF in the culture supernatant. After incubation with AB8.28–Sepharose, the majority of LGL cells expressed either monomorphic DR or DQ determinants, along with TAC, thus demonstrating by multiple phenotypic criteria that they were in an activated state. Next, we focused our attention on the release by AB8.28-activated LGL of the soluble mediator PAF. The appearance of human 1a paralleled the release of a significant amount of PAF. The latter event was further confirmed by treating resting LGL with A23187, a stimulus that bypasses the receptor and activates a specific acetyltransferase necessary for PAF biosynthesis (20). Maximal PAF release occurred 45 min after challenge with AB8.28–Sepharose and A23187. PAF released by A23187-treated LGL was almost half the amount obtained from an analogous concentration of PMN, even if this may be an underestimate, since the long purification might reduce
LGL potential for PAF biosynthesis. The lipid material released from LGL shared with synthetic 1-O-octadecyl-2-acetyl-sn-glycerol-3-phosphocholine the physical and chemical characteristics (21), the retention time on HPLC, the sensitivity to phospholipase A$_2$ (17), and biological activity on rabbit platelets (22). The enhancement of PAF release in the presence of Ac-CoA suggested an acetylation process in the PAF biosynthesis (14). The specificity of PAF release upon FcR stimulation was demonstrated by the fact that Sepharose coated with class-matched IgG from HLA class I (positive control) and class II (negative control) mAbs did not induce PAF release. Furthermore, monocytes did not release PAF upon FcR triggering, whereas PMN released substantially lower amounts. This is in line with the observation that PMN challenged with IgG-coated yeast particles release PAF (22).

In conclusion, binding of mAb AB8.28 induces LGL activation, an event that can be monitored through the expression of phenotypic markers and the release of detectable amounts of PAF. The importance of these observations is 3-fold.

(i) Mature resting NK cells share with T and B lymphocytes the ability to be activated in response to appropriate stimuli such as interferon in non-human systems (23), IL-2 (24), and—as shown here—FcR challenge. London (25) has shown recently that purified PBL NK cells can be induced to proliferate in short-term cultures by medium containing IL-2 and irradiated allogeneic lymphoblastoid cells. The activation antigens expressed after treatment with IL-2-conditioned medium include those observed after FcR triggering. This suggests that nonadaptive immunity may be activated through different mechanisms, which include a multipurpose humoral factor such as IL-2 and a more rigid system mediated through the FcR. The importance of this molecule as a signal triggering cell–cell interactions is further emphasized by the observation that a mixed lymphocyte culture (MLC) test is able to induce the expression of the AB8.28 molecule on a population of resting T cells, which are AB8.28$^-$. These observations (26) are in full agreement with those reported by Phillips et al. (27) and are probably due to expansion of a residual small PBL subset, a vestigium of a population active during fetal life: in fact, mononuclear cells from fetal (16 week) thymus are AB8.28$^+$ (28).

(ii) LGL are a potent source of mediators such as interleukin 1 (29), leukotrienes (30), NK cytotoxic factor (31), and—as demonstrated here—PAF. All of these factors could play a significant role in the NK toxic mechanism(s) or in cell–cell interactions (5), as suggested by Hoffman et al. (6), who showed that PAF and its analogues (in high concentrations) are cytotoxic for human cultured cells. Furthermore, LTB4, an activator of other cell systems such as PMN, may lead to the release of postulated cytotoxic factors (32) such as PAF.

(iii) The expression of class II antigens may prompt revision of the view that NK function is fully HLA-independent. A working hypothesis is that activation by way of FcR is a physiological event that induces human Ia expression during cell–cell interactions. The inability to detect expression of class II antigens in all LGL may reflect the inadequacy of the stimulus or of the experimental conditions. In keeping with this possibility is the observation that soluble mAbs are unable to trigger LGL. This may be due to the extremely brief duration of the triggering event(s) mediated by soluble antibody, which cannot be recorded.

To our knowledge, it has not been reported previously that LGL induction upon FcR triggering is allowed by LGL activation and expression of DR/DQ molecules and IL-2 receptor on the cell surface along with the release of PAF, a mediator of cell–cell communication.

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