Intimate Cell Conjugate Formation and Exchange of Membrane Lipids Precede Apoptosis Induction in Target Cells during Antibody-Dependent, Granulocyte-Mediated Cytotoxicity

Heike Horner,* Carola Frank,* Claudia Dechant,† Roland Repp,‡ Martin Glennie,§ Martin Herrmann,2,3† and Bernhard Stockmeyer2*

Ab-dependent polymorphonuclear granulocyte (PMN)-mediated cytotoxicity may play an important role in the control of malignant diseases. However, little is known as to which particular pathways are used for the killing of malignant cells by PMN. The production of reactive oxygen intermediates (ROI) has been observed to occur during Ab-dependent, cell-mediated cytotoxicity (ADCC). However, PMN from a patient with chronic granulomatous disease demonstrated strong ADCC against malignant lymphoma cells. Furthermore, the inhibition of ROI production in PMN from healthy donors had no significant effect on ADCC. Therefore, ROI production by the NADPH oxidase of PMN does not appear to be mandatory for PMN-mediated ADCC. Recent data suggest a role for perforins in PMN-mediated cytotoxicity. However, in our assays concanamycin A, an inhibitor of perforin-mediated ADCC by mononuclear cells, had no inhibitory effect on PMN-mediated ADCC. Using electron microscopy we observed that PMN and their target cells intimately interact with the formation of interdigitating membrane protrusions. During PMN and target cell contact there was a mutual exchange of fluorescent membrane lipid dyes that was strongly increased in the presence of tumor-targeting Abs. This observation may be closely related to the recently described process of trogocytosis by lymphocytes. The presence of transient PMN-tumor cell aggregates and the accumulation of PMN with tumor cell-derived membrane lipids and vice versa were associated with effective ADCC as measured by chromium-release or apoptosis induction. The Journal of Immunology, 2007, 179: 337–345.

The capacity to mediate Ab-dependent cell-mediated cytotoxicity (ADCC) has been demonstrated in vitro for monocytes/macrophages and NK cells as well as for polymorphonuclear granulocytes (PMN). PMN are potent effector cells against a wide range of malignancies in vitro (1–3) and are considered important for the rejection of malignant tumors in vivo (4). In contrast to T cells, the cytotoxicity of PMN is dependent on Ab binding to the target cells. PMN from G-CSF-treated patients were the predominant effectors for the killing of breast cancer cells in vitro in the presence of bispecific Ab (bsAb), recognizing both FcγRI and tumor target Ag HER-2/neu (2). Recent therapeutic advances in Ab-mediated cancer therapy (5, 6) have renewed the interest in PMN-mediated ADCC. However, the mechanism of target cell death remains elusive (7). Usually, PMN-mediated ADCC has been measured by 3 h of 51Cr-release assays (8, 9) with high E:T cell ratios (10). Previous experiments with PMN effectors demonstrated unexplained differences in tumor cell-related Ag serving as targets for ADCC. Thus, mAb against HLA class II or against members of the epidermal growth factor receptor family such as HER-2/neu were highly effective in recruiting PMN as effector cells, whereas CD10, CD19, CD21, CD37, and CD38 Ab were ineffective in this regard (1–3, 11, 12).

To recruit cell-mediated effector mechanisms, Ab must interact with Ig Fc receptors, which are classified as Fcγ, Fcα, or Fcε receptors depending on their specificity for IgG, IgA, or IgE, respectively (13). The pivotal role of Fc receptors for the antitumor activities of mAb in vivo has been demonstrated in knockout mice in which the signaling machinery of the Fc receptors had been genetically disrupted (14). Furthermore, recent studies of non-Hodgkin’s lymphoma showed a correlation between the FcγRIIA (CD16a) and FcγRIIa (CD32) genotype and the clinical and molecular responses to a CD20-directed therapy with the mAb rituximab (15–17). However, conflicting results have been reported during the investigation of other malignancies treated with mAb (18).

Received for publication June 2, 2006. Accepted for publication April 14, 2007. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Wilhelm Sander-Stiftung (Grant 2000.032.2), the Interdisciplinary Center for Clinical Research (Projects A3 and A4), the ELAN-Fond (06.05.21.41) at the University Hospital of the University of Erlangen-Nuremberg, Deutsche Forschungsgemeinschaft Sonderforschungsbereich 643 (project B5), and the Lupus Erythematosides Selbsthilfe-gemeinschaft, eingetragener Verein.

2 M.H. and B.S. contributed equally to the senior authorship.

3 Address correspondence to Dr. Martin Herrmann, Institute for Clinical Immunology, Department of Internal Medicine 3, Friedrich-Alexander University of Erlangen-Nuremberg, Krankenhausstrasse 12, 91054 Erlangen, Germany. E-mail: Martin.Herrmann@med3.imed.uni-erlangen.de

4 Abbreviations used in this paper: ADCC, Ab-dependent cell-mediated cytotoxicity; bsAb, bispecific Ab; CGD, chronic granulomatous disease; CMA, concanamycin A; DIL, 2-[3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-yliden)-1-propenyl]-3,3-dimethyl-1-octadecyl-perchlorate; DIo, 3-octadecyl-2-[3-(3-octadecyl-2,3H)-benzoazolyldiene]-1-propenyl-perchlorate; DPL, diphenyleneiodonium chloride; FSC, forward scatter; IgG1, humanized IgG1; mlgG1, mouse IgG1; MNC, mononuclear cell; NHD, normal healthy donor; PI, propidium iodide; PMN, polymorphonuclear granulocyte; Raji, HER-2/neu transfected Raji cell; ROI, reactive oxygen intermediate; SSC, side scatter; z-VAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00.

www.jimmunol.org
PMN constitutively express FcγRI (CD89), the low affinity FcγRIIa (CD32a), and FcγRIIIb (CD16b) (19). The expression of the high-affinity FcγRI (CD64) can be induced in vivo by stimulation with G-CSF (20). We have recently demonstrated that FcγRI-directed bsAb proved more effective in triggering tumor cell lysis than the respective Fcγ-directed constructs, particularly with unstimulated PMN (19, 21). In contrast to some members of the FcγR family, FcγRI is exclusively expressed on cytotoxic cells (22).

A breakdown of the membrane lipid asymmetry, the loss of matrix adhesion and mitochondrial membrane potential, the “boiling” of cytoplasm, the condensation of chromatin, and finally, the internucleosomal cleavage of the nuclear DNA characterize apoptotic cell death (23). At an early stage of apoptosis the exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane is detected by the binding of annexin V. DNA fragmentation and the loss of chromatin resulting in a sub-G1 DNA content of the dying cells is a characteristic feature of the later stages of apoptosis. Under physiological conditions when high E:T cell ratios are unlikely to occur, apoptosis induction is supposed to be the major pathway of cell death (7). After activation, PMN produce reactive oxygen intermediates when high E:T cell ratios are unlikely to occur, apoptotic effects, but their role in ADCC remains to be defined.

Table I. Percentage of double fluorescent cells in populations 1, 2, and 3 presented as mean ± SEM of 16 independent experiments

| Measured Parameter      | 0 min | 30 min | 90 min |
|-------------------------|-------|--------|--------|
| Population 1 (PMN)      | 0.73±0.18 | 1.25±0.26a | 4.09±0.73a | 1.15±0.63 | 5.71±0.91ab | 26.31±2.24ab |
| Population 2 (Raji HER-2/neu) | 0.09±0.02 | 0.49±0.15 | 3.45±0.94 | 0.08±0.01 | 3.00±0.83ab | 4.61±1.42a  |
| Population 3 (Aggregates) | 0.67±0.15 | 0.67±0.09 | 0.88±0.12 | 0.69±0.20 | 7.10±0.73ab | 8.20±0.82ab |

*Significantly different from respective population at time point 0 (p < 0.05).
*Significantly different from respective population incubated without bsAb at the same time point (p < 0.05).

187.1 (expressing mAb against mouse L chain; HB-58), were obtained from the American Type Culture Collection. The HER-2/neu transfected Raji cell line (Rajiβ) was generated as described elsewhere (30). Cells were kept in RF10 medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 3 mM L-glutamine (Invitrogen Life Technologies).

**Isolation of effector cells**

The experiments reported here were conducted in accordance with the Declaration of Helsinki. After informed consent, 10–20 ml of citrate anticoagulated peripheral blood was drawn from normal healthy donors (NHD) or a patient with chronic granulomatous disease (CGD) for effector cell preparation. The male CGD patient had an X-linked deletion of CI475 in exon 12 of the CYBB gene encoding gp91phox, predicting a frame shift and resulting in a stop codon in the NADPH binding domain (31). PMN were isolated from peripheral blood as described (11). Briefly, citrate anticoagulated blood was layered over a discontinuous Percoll gradient (62 and 70%; Seromed). After centrifugation, PMN were collected at the interface between the two Percoll layers. The remaining erythrocytes were removed by hypotonic lysis. The purity of PMN as determined by cytospin preparations exceeded 95%, and cell viability was >95% as tested by trypan blue exclusion. Mononuclear cells (MNC) containing NK cells, T cells, B cells, and monocytes/macrophages were collected between the plasma and the 62% Percoll layer.

**ADCC assays**

Chromium 51 release assays were performed as described elsewhere (11). Briefly, target cells were labeled with 200 μCi of 51Cr for 2 h. To investigate the influence of caspases on PMN-mediated ADCC, Rajiβ cells were preincubated with 100 μM z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk; Alexis), a pan-caspase inhibitor, during the labeling process with chromium. After extensive washing with the RF10 medium, cells were adjusted to 105/ml. PMN or MNC at an E:T ratio of 40:1 and sensitizing Ab at final concentrations of 2 μg/ml were added to round-bottom microtiter plates (Nunc) unless otherwise noted. To address the role of ROI production in ADCC of Rajiβ, PMN were preincubated with 25 μM di-phenyllelenodiocin chloride (DPI; Sigma-Aldrich), an inhibitor of neutrophil NADPH oxidase (32), for 5 min at room temperature and added to assays without washing, giving a final concentration of DPI of 6.3 μM. Assays were performed in RF10 medium in the presence of 50 U/ml GM-CSF and started by adding 50 μl of chromium-labeled target cells, resulting in a final volume of 200 μl. After 3 h of incubation (37°C with 5% CO2), assays were stopped by centrifugation, and 51Cr release from triplicate samples was measured. The percentage of cellular cytotoxicity was calculated using the following formula: specific lysis = ([experimental cpm – basal cpm] / [maximal cpm – basal cpm]) × 100%

Maximal 51Cr-release was determined by adding perchloric acid to target cells at a final concentration of 3%. Basal release was measured in the absence of sensitizing Ab and effector cells. No Ab-independent cytotoxicity was observed with PMN as effector cells.

**Oxidative burst assays**

The luminol-ECL method was used for the detection of total (intracellular and extracellular) ROI production. PMN (4 x 105) from NHD were gently centrifuged (40 x g for 3 min at 4°C) on targets (1 x 105) Rajiβ cells opsonized with or without Ab (2 μg/ml) in PBS supplemented with 10% FCS and 8.8 mM glucose. To inhibit ROI production, PMN from NHD were preincubated with 25 μM DPI for 5 min at room temperature and added to assays without washing, giving a final concentration of DPI of 5.7 μM. GM-CSF (50 U/ml) and luminol (50 μM) were added, giving a final volume of 220 μl. Luminol-ECL was measured in triplicates as counts per minute.
second in a Centro LB 960 microplate luminometer (Berthold Technologies). For maximal oxidative burst, PMN were stimulated with 150 nM phorbol myristate acetate (data not shown).

Electron microscopy

Transmission electron microscopy was performed as described elsewhere (33). Briefly, 187.1 rat hybridoma cells sorted for high membrane expression of a mAb against a mouse \( \kappa \) L chain and freshly isolated PMN from a patient treated with G-CSF were incubated at an E:T ratio of 5:1 with or without 2 \( \mu \text{g/ml} \) mAb 22 (mIgG1), retargeting FcRI on effector cells. Incubation was stopped by adding cold Ito’s fixative. The cell suspension was transferred on 6.5-mm diameter, 0.4-\( \mu \text{m} \) pore-size Transwell filters (Costar) and fixed for 4 h at 4°C. Subsequently, filters were postfixed in reduced osmium, encapsulated in 2% agar, stained with uranyl acetate, dehydrated in a graded series of ethanolic solutions completed by pure acetone, and finally embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a Zeiss EM T109 transmission electron microscope.

E:T cell aggregate formation assays

The Vybrant lipophilic carbocyanine dyes 2-[3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidine)]-1-propenyl]-3,3-dimethyl-1-octadecyl-perchlorate (DiO; V22886) and 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoazolylidene)]-1-propenyl]-perchlorate (DiI; V22886) (Molecular Probes) are used for long-term labeling and tracking of cells (34). They are fluorochromes emitting light with a fluorescence emission maximum of 566 and 501 nm, respectively. Cells that formed aggregates can be identified by forward scatter (FSC)/side scatter (SSC) characteristics and double fluorescence. Effector cells were stained in a solution of DiO (1 \( \mu \text{M} \)) and target cells were stained in a solution of DiI (2 \( \mu \text{M} \)) for 15 min at 37°C and protected from light. After washing three times with PBS, fixed with 3% paraformaldehyde, and embedded in a drop of Aquatex (Merck). Laser scanning microscopy was done with a Leica TCS SP2 instrument (Leica Microsystems) using an argon laser for blue light (488 nm) excitation and a red light-emitting helium-neon laser (543 nm) for DiO and DiI, respectively.

Apoptosis assays

For the measurement of programmed cell death, a previously described method was adapted (35). RajiH target cells at 10\(^6\) per ml were stained for

![FIGURE 1. PMN-mediated ADCC. A, Lysis of RajiH via various target Ags. Significant cytotoxicity was achieved by targeting HLA class II with mAb F3.3 or HER-2/neu with the bsAb [A77 \( \times \) trastuzumab] with PMN from NHD as effectors. No significant killing was observed by targeting CD19 with mAb 4G7c. B and C, Effect of Ab concentration (D) or E:T ratio (C) on HLA class II-directed (F3.3; \( \triangle \)) or HER-2/neu-directed ([A77 \( \times \) trastuzumab]; \( \square \)) PMN-mediated cytotoxicity. Significant ADCC was induced starting from 0.08 \( \mu \text{g/ml} \) for HLA class II mAb or 0.4 \( \mu \text{g/ml} \) for HER-2/neu bsAb. Significant \(^{51}\text{Cr} \) release occurred from an E:T ratio of 10:1. D, Oxidative burst activity of PMN was observed in the presence of mAb F3.3 (\( \Delta \)) or bsAb [A77 \( \times \) trastuzumab] (\( \square \)]. Marginal ROI production was detected in the presence of mAb 4G7c (\( \odot \)) or the absence of sensitizing Ab (\( \bullet \)). ROI production was measured at an E:T ratio of 2.5:1. Experiments from 29 (A) or three NHD (B–D) are presented as mean \( \pm \) SEM. * \( p < 0.05; \) significant killing.

![FIGURE 2. ADCC and oxidative burst activity of isolated PMN from a CGD patient (open bars and symbols) or NHD (filled bars and symbols) against RajiH. A, Effector cells from a CGD patient or a NHD mediated similar ADCC by targeting HLA class II with mAb F3.3 or HER-2/neu with bsAb [A77 \( \times \) trastuzumab]. Three experiments are shown. B, Oxidative burst capacity of PMN from NHD or the CGD patient was measured by chemiluminescence. Efficient ROI release was detectable for NHD in the presence of mAb F3.3 (triangles) or bsAb [A77 \( \times \) trastuzumab] (squares), whereas the PMN of CGD did not produce detectable amounts of ROI. Mean \( \pm \) SEM of triplicates from one representative experiment of two is shown.
10 min at 37°C and 5% CO2 with 2μM CFSE, which covalently binds to nuclear proteins. CFSE remains stably associated with the nuclei of cells even after hypotonic lysis by detergent. In this way we were able to differentiate the CFSE-stained Raji nuclei from the unlabeled PMN nuclei during FACS analysis. Staining was terminated by adding FCS to a final concentration of 10%. After 4 h of culture at 37°C and 5% CO2 for the diffusion of unconjugated reagent and by-products, cells were washed twice with ice-cold PBS. Cells were resuspended at 10^6/ml in RF10 medium and isolated PMN were added to obtain an E:T ratio of 2.5 or 20:1 in the presence of 50 U/ml GM-CSF or without the bsAb [A77 × trastuzumab]. After 6, 24, and 40 h, 100 μl of coculture was treated with 500 μl of hypotonic propidium iodine (PI) staining solution (0.1% sodium citrate, 0.1% Triton X-100, and 1 μg/ml PI) and incubated at 4°C in the dark for 24 h before measurement. Flow cytometric analysis of CFSE and PI-labeled nuclei and fragments from triplicate samples was performed on an EPICS Profile flow cytometer.

**Statistical analysis**

Results are expressed as means ± SEM. Statistics were performed with ANOVA, Levene’s test for homogeneity of variance, and Dunnett’s post hoc test or paired Student’s t test where appropriate (SPSS 14.0.1 for Windows).

**Results**

The target Ag and the Ab contribute to effective PMN-mediated ADCC

Ab directed against the target Ags HER-2/neu, HLA class II, or CD19 were analyzed for their ability to induce ADCC of RajiH cells by PMN. As shown in Fig. 1A, PMN-mediated lysis is restricted to distinct target Ags. Significant cytotoxicity occurred with Ab against the target Ags HLA class II and HER-2/neu. Consistent with our previously published data, PMN failed to induce ADCC in the absence of Ab or in the presence of the mAb 4G7c against CD19 (11, 30), although the Ag density of HER-2/neu (9.9 ± 1.7, mean fluorescence intensity ± SEM) and CD19 (5.3 ± 1.3, mean fluorescence intensity ± SEM) was in a similar range (n = 10, p = 0.2).

The HLA class II-directed mAb F3.3 demonstrated significant tumor cell killing, starting from 0.08 μg/ml to 10 μg/ml (Fig. 1B).
FIGURE 5. Ab-induced effector-target cell aggregation formation. Dil (FL2)-labeled RajiH and DiO (FL1)-labeled PMN were cocultured at an E:T ratio of 2.5:1 as indicated. In the FSC/SSC dot plots, population 1 (low FSC/high SSC) depicts PMN, population 2 (low FSC/low SSC) depicts target cells, and events in population 3 (high FSC/high SSC log) are supposed to be effector-target cell aggregates. Each population was further analyzed in FL-1/FL-2 dot plots. In the presence of bsAb, double positive single PMN and target cells were detected (C and E, columns 1 and 2 of FL1/FL2 dot plots), suggesting mutual membrane dye transfer. FL1/FL2 double positive fluorescent events in population 3 were increased in the presence of bsAb [A77 × trastuzumab], suggesting the induction of aggregate formation by bsAb (C and E). In the absence of bsAb, RajiH and PMN showed more of a tendency for homotypic than heterotypic aggregation (A, B, and D, column 3 of FL1/FL2 dot plots). One representative evaluation of 16 is shown.

With the bsAb [A77 × trastuzumab] recognizing FcRI and HER-2/neu, cytotoxicity was observed starting from 0.4 µg/ml to 10 µg/ml, reaching an optimum at 2 µg/ml (Fig. 1B). In both cases specific lysis was found starting from an E:T ratio as low as 10 to 1 (Fig. 1C). In summary, the target Ag, the Ab concentration, and the E:T ratio all play important roles in determining the efficiency of the PMN-mediated ADCC of RajiH cells.

**ROI production is not mandatory for PMN-mediated ADCC**

The generation of ROI during ADCC against RajiH1 was monitored by luminol-amplified chemiluminescence (36). We observed Ab-dependent ROI production of PMN from NHD with Abs targeting HER-2/neu ([A77 × trastuzumab]) or HLA class II (F3.3), but not with the mAb against CD19 (4G7c), which did not mediate ADCC (Fig. 1D). This suggests an involvement of ROI in PMN-mediated ADCC. However, the Ab that mediated the most efficient cytotoxicity (the classical mAb against HLA class II) was not the Ab that induced the highest ROI production (the bsAb using the FcRII of PMN and HER-2/neu on target cells). Therefore, ROI production did not strictly correlate with ADCC activity.

To further investigate the role of ROI production, we compared the PMN from a patient suffering from CGD with the PMN from a NHD in regard to ADCC with RajiH1 cells. CGD is characterized by the complete lack of ROI generation and, consequently, of oxidative burst activity. As shown in Fig. 2A, the PMN of both the CGD patient and the NHD efficiently killed tumor targets in the presence of the HLA class II-directed mAb or the HER-2/neu-directed bsAb. Efficient ROI production was observed with PMN from the NHD stimulated by F3.3- or [A77 × trastuzumab]-coated targets in contrast to the PMN from the CGD patient, which were unable to produce ROI (Fig. 2B). These results were further supported by the inhibition of the neutrophil NADPH oxidase of PMN from the NHD with DPI, which resulted in a ≥95% reduction of ROI production. This had no significant effect on the PMN-mediated specific lysis of RajiH1 compared with untreated PMN (n = 6) by targeting HLA class II (47 ± 9 and 57 ± 6%, respectively) or HER-2/neu (24 ± 4 and 26 ± 6%, respectively). Therefore, ROI production by the NADPH oxidase of PMN does not appear to be mandatory for the PMN-mediated lysis of RajiH1 cells.

**The perforin inhibitor CMA does not inhibit PMN-mediated ADCC**

As previously reported, CMA inhibits the perforin-based pathway of NK cell- and cytotoxic T cell-mediated cytolyis (37). Furthermore, recent data suggest a role for perforins and granzymes in PMN-mediated cytotoxicity (25). On the basis of these findings, we tried to elucidate the role of perforins in PMN-mediated ADCC by inhibition of the perforin-based pathway with CMA (Fig. 3). We used PMN and MNC as effectors targeting the B cell lymphoma ARH-77 via HLA class II, the breast cancer cell line SK-BR-3 via HER-2/neu, and RajiH1 cells via both HLA class II and HER-2/neu. In the presence of CMA, PMN-mediated lysis of ARH-77 via HLA class II was significantly enhanced using the mAb F3.3 (Fig. 3A). No inhibition by CMA of PMN-mediated ADCC was observed for SK-BR-3 onopsonized with the bsAb [A77 × trastuzumab] (Fig. 3C). The PMN-mediated ADCC of RajiH1 cells remained unaffected by CMA by the targeting of HER-2/neu or HLA class II with bsAb [A77 × trastuzumab] and mAb F3.3, respectively (Fig. 3E). In contrast, perforin-mediated ADCC by MNC was significantly inhibited in the presence of CMA for both target Ags (Fig. 3, B, D, and F). Therefore, we conclude that PMN-mediated ADCC is not dependent on the perforin pathway.

**Aggregate formation and membrane dye exchange is induced by Ab**

To initiate ADCC, PMN have to adhere to their target cells. As shown by electron microscopy, PMN made intimate contacts with Ab-opsonized tumor cells (Fig. 4, A–E). Importantly, PMN did not adhere to target cells in the absence of an opsonizing Ab (Fig. 4F), indicating that an Ab is required for aggregate formation. These
data were further supported by flow cytometry showing clusters between PMN and tumor cells. We adapted a commonly used assay for the quantification of aggregates (38). The plasma membranes of PMN effector and RajiH cells were labeled with the fluorescent carbocyanines DiO or DiI, respectively. Labeled cells were cocultured in the presence or absence of a sensitizing Ab and analyzed for cluster formation at the time points 0, 30, and 90 min (Table I and Fig. 5). In FSC/SSC dot plots three populations were to be observed. Population 1 with high SSC and low FSC and population 2 with low SSC and FSC represent PMN and RajiH cells, respectively. Clustered cells were found in population 3 characterized by their increased FSC/SSC. After coculture for 30 or 90 min, an ~10-fold increase in the number of aggregates was found in the presence of bsAb, whereas no significant cluster formation was to be observed without a bsAb (Table I). In addition to the aggregates, after 30 min significantly more double fluorescent PMN (population 1) or target cells (population 2) appeared in cocultures with bsAb compared with assays without bsAb. The percentage of double fluorescent PMN further significantly increased from 30 to 90 min, indicating an ongoing recruitment of PMN for ADCC. A small increase of double fluorescent cells was also observed for PMN and RajiH independently of bsAb (Table I and Fig. 5).

In Fig. 5 we show a more detailed evaluation of the cocultures. High percentages of double fluorescent PMN-target cell clusters (92–96%) were to be observed in population 3 of the aggregated cells in the presence of bsAb [A77 × trastuzumab] (Fig. 5, C and E). Cocultures without bsAb displayed only low percentages of double fluorescent cell clusters. Furthermore, both RajiH and PMN showed a tendency for homotypic aggregation in the absence of bsAb (Fig. 5, A, B, and D). Interestingly, the single effector cells (population 1) and the target cells (population 2) contained more double fluorescent cells after coculture in the presence of bsAb (Fig. 5, C and E) than without bsAb (Fig. 5, B and D).

The time course of effector-target cell clustering analyzed by flow cytometry is shown in Fig. 6. For this purpose, labeled cells were cocultured in the presence of the bsAb [A77 × trastuzumab] at 37°C and the double fluorescent cells in each population were repeatedly measured at short time intervals. Directly after mixing, double fluorescent cell clusters were detected. After reaching a maximum between 30 and 60 min, the number of double fluorescent clusters slowly decreased. In contrast, double fluorescent single effector or target cells were <1% for the first 10 min of coculture. Subsequently, the number of double fluorescent PMN and target cells increased. The percentage of double fluorescent target cells (population 2) remained stable and lower than that observed for
FIGURE 8. Apoptosis induction by the bsAb [A77 × trastuzumab]. CFSE-labeled Raji^{H} cells were cocultured with unlabeled PMN in the presence (●) or absence (●) of the bsAb [A77 × trastuzumab] at an E:T ratio of 2.5:1 (dashed line) or 20:1 (solid line). After 6, 24, or 40 h of coculture the cells were stained with PI-Triton X-100. Apoptosis of target cells was analyzed as CFSE/PI double-labeled nuclear fragments with subG1 DNA content. After 24 or 40 h significant apoptosis was measured in assays with bs/Ab compared with assays without bs/Ab (*, p < 0.05), and significantly more apoptosis was detectable at an E:T ratio of 20:1 compared with an E:T ratio of 2.5:1 (#, p < 0.05). Analyzes were performed from triplicates.

PMN. Coculture at 4°C revealed bs/Ab-dependent formation of cell clusters, but no membrane dye exchange occurred (not shown). We conclude that heterotypic clustering precedes mutual membrane lipid exchange between effector and target cells during ADCC.

To visualize aggregate formation and membrane dye exchange, confocal laser scanning microscopy was performed (Fig. 7). Dil-labeled Raji^{H} and DiO-labeled PMN were detected as red and green cells, respectively. Labeled PMN and Raji^{H} cells that had mutually exchanged fluorescent membrane lipids appeared yellow in the merged image. Morphologically, effector and target cells could be distinguished by their characteristic nuclear features. An increased number of yellow cells were found in the presence of the bsAb [A77 × trastuzumab] compared with control experiments without Ab. Therefore, membrane lipid exchange dramatically in the presence of an Ab.

Apoptosis is induced in PMN-mediated ADCC

To elucidate the mechanism of Ab-mediated Raji^{H} cell death by PMN, we adopted a previously described method for the detection of apoptosis (35). Raji^{H} cells were stained with CFSE, washed, and mixed with isolated PMN. CFSE remains stably associated with the nuclei of cells even after hypotonic lysis. In this way, we were able to differentiate the CFSE-stained Raji^{H} nuclei from unlabeled PMN nuclei during FACS analysis. After 6, 24, and 40 h of coculture in the presence or absence of the bsAb [A77 × trastuzumab], cells were stained with hypotonic PI-Triton X-100. The PI fluorescence of CFSE-tagged nuclei was measured and CFSE-positive nuclei with sub-G1 DNA content were considered being apoptotic target cell nuclei. In ADCC assays with an E:T ratio as low as 2.5:1 significant apoptosis was detected in the presence of the bsAb [A77 × trastuzumab] 24 and 40 h after coculture. Apoptosis was even more pronounced in assays with an E:T ratio of 20:1 (Fig. 8). Simultaneously, the number of intact target cell nuclei (G_{0}-G_{1}) decreased. As expected for GM-CSF-exposed PMN, apoptosis ranged from 30 to 50% from 24 to 40 h irrespective of the presence of bsAb. We found no indication for the aggregation of nuclear fragments from osmotically lysed apoptotic Raji^{H} cells or PMN. If we had not been able to differentiate apoptotic effector and target cell nuclei we would expect to observe much higher levels of apoptotic Raji^{H} nuclei. In long term culture for 6 days, medium consumption of Raji^{H} cells ceased in the presence of bsAb at an E:T ratio of 20:1, indicating almost complete target cell death.

The mechanism of apoptosis in PMN-mediated ADCC was further investigated with the irreversible pan-caspase inhibitor z-VAD-fmk. Targeting HER-2/neu, z-VAD-fmk treatment of Raji^{H} significantly reduced PMN-mediated lysis of Raji^{H} compared with control (51 ± 3 and 31 ± 4%, respectively; p = 0.00003, n = 6). Interestingly, z-VAD-fmk had no effect on HLA class II-directed ADCC compared with control (91 ± 5 and 90 ± 5 respectively; n = 6). Treatment of isolated PMN with z-VAD-fmk did not affect ADCC.

Discussion

Several factors affect the efficiency of PMN in ADCC. Thus, the specificity, concentration, and format of Ab, as well as E:T ratio, density, and domains of target Ag, contribute substantially to effective Ab-mediated tumor cell killing by PMN (3, 7, 11, 12, 19, 21, 30). As previously shown, HER-2/neu and HLA class II proved to be appropriate target Ag of PMN-mediated lysis of Raji^{H} cells. In contrast, no ADCC was mediated via the CD19 Ag despite a similar surface density to HER-2/neu (30). To get insight into the mechanisms of ADCC, we used Raji^{H} cells as targets and freshly isolated PMN as effector cells. We demonstrated efficient recruitment of effectors by HER-2/neu-directed Fab’{2} bsAb or HLA class II-directed IgG mAb at low E:T ratios and Ab concentrations.

Activated PMN produce ROI; however, there are contradictory reports about the role of ROI in PMN-mediated ADCC (39, 40). ROI production of PMN from NHD was triggered by mAb to HLA class II or bsAb to HER-2/neu, but not by CD19 mAb. This observation may implicate an Ag restriction in oxidative burst activity as previously described for ADCC (11). However, ROI production did not strictly correlate with ADCC activity. Thus, the oxidative burst targeting of HER-2/neu with bsAb was clearly stronger than the targeting of HLA class II with IgG mAb, whereas the relative effectiveness of ADCC was vice versa. More important, PMN from a CGD patient completely lacking ROI production mediated equally efficient ADCC like the PMN from NHD targeting HER-2/neu and HLA class II. Furthermore, the inhibition of ROI production in PMN from NHD had no effect on ADCC against Raji^{H} cells. These data are in accordance with data from Katz et al. (39) that demonstrated a role for ROI in ADCC exclusively against erythrocytes but not against lymphoid target cells. From our experiments we conclude that NADPH-dependent ROI production is not mandatory for PMN-mediated ADCC against Raji^{H}.

To elucidate the contribution of perforin to PMN-mediated ADCC we used CMA, an inhibitor of the vacuolar type H^{+} ATPase of perforin-based cytotoxicity. CMA inhibits the acidification of lytic granules and the activation of perforin in NK cells, resulting in reduced NK cell-mediated cytotoxicity (28). However, in experiments with PMN from NHD as effector cells, we found that CMA had no or even a stimulatory effect on PMN-mediated ADCC (Fig. 3). CMA inhibits the acidification of lytic granules and the activation of perforin in NK cells, resulting in reduced NK cell-mediated cytotoxicity (28). However, in experiments with PMN from NHD as effector cells, we found that CMA had no or even a stimulatory effect on PMN-mediated ADCC (Fig. 3). CMA inhibits the acidification of lytic granules and the activation of perforin in NK cells, resulting in reduced NK cell-mediated cytotoxicity (28). However, in experiments with PMN from NHD as effector cells, we found that CMA had no or even a stimulatory effect on PMN-mediated ADCC (Fig. 3). CMA inhibits the acidification of lytic granules and the activation of perforin in NK cells, resulting in reduced NK cell-mediated cytotoxicity (28). However, in experiments with PMN from NHD as effector cells, we found that CMA had no or even a stimulatory effect on PMN-mediated ADCC (Fig. 3). CMA inhibits the acidification of lytic granules and the activation of perforin in NK cells, resulting in reduced NK cell-mediated cytotoxicity (28). However, in experiments with PMN from NHD as effector cells, we found that CMA had no or even a stimulatory effect on PMN-mediated ADCC (Fig. 3). CMA inhibits the acidification of lytic granules and the activation of perforin in NK cells, resulting in reduced NK cell-mediated cytotoxicity (28). However, in experiments with PMN from NHD as effector cells, we found that CMA had no or even a stimulatory effect on PMN-mediated ADCC (Fig. 3).
during intimate contact formation we observed cytoplasmic communication between effector and target cells (Figs. 4, D and E) in the presence of mAb. This cytoplasmic communication may specifically characterize the “immunological synapse” formed between PMN and target cells during ADCC, which will be referred to as the “cytotoxic synapse.” β3 integrins are described to be necessary for the formation of the immunological synapse and to redistribute into membrane rafts. In this way they may isolate the small cytoplasmic communications we have observed from the remaining cell membrane, preventing the progression to cell fusion. Additionally, in our experiments video-assisted microscopy of living cells (not shown) and confocal laser-scanning microscopy revealed the formation of target-effector cell conjugates and enhanced membrane dye transfer in the presence of bsAb (Fig. 7) without evidence of cell-cell fusion.

Transient aggregate formation between effector and target cells was detected by flow cytometry immediately after mixing in the absence of bsAb. This observation suggests that cells make rapid contacts by accidental collision. In the presence of bsAb persistent aggregate formation was observed (Fig. 6). Thus, the bsAb targeting FcαRI and HER-2/neu establishes a long-lasting tethering between effector and target cells by interaction with the Fc receptor and the tumor cell Ag, respectively. Adhesion molecules such as integrins on the PMN and intercellular adhesion molecules on the surface of tumor cells may further contribute to the stable formation of the “cytotoxic synapse” (36, 43). In the absence of bsAb we detected homotypically aggregated RajiH cells (population 3, upper left quadrants in Fig. 5, A–C) that have been described as adhering to each other and exchanging membrane fragments spontaneously (44). After bsAb-mediated adhesion of PMN to RajiH, heterotypic aggregates formed and the number of exclusively Dil-positive aggregated RajiH cells decreased. From our data we cannot determine whether PMN disrupted the homotypic aggregates of RajiH cells to form heterotypic aggregates or whether PMN were recruited to the homotypic aggregates of RajiH.

Our data demonstrate a small increase in the number of double fluorescent solitary PMN and RajiH over time that is independent of bsAb, implying membrane dye exchange during some random collisions between effector and target cells (Figs. 5 and 6 and Table I). However, in the presence of bsAb significantly more double fluorescent single PMN or RajiH cells were to be detected. This may be explained by: 1) prolonged contacts between effector and target cells as shown for retargeted T cells (45); 2) the generation of chemoattractants for PMN by tumor cells after opsonization with bsAb; or 3) the activation of the mobility or adhesiveness of the PMN by triggering FcαRI with bsAb. For RajiH, a significant bsAb-dependent uptake of membrane dye from PMN was observed at 30 min but not at 90 min (Table I). This most likely reflects [A77 × trastuzumab]-mediated early target cell death, which was previously shown to require the interaction between the Fc receptors on PMN and target cells for the induction of apoptosis (7). As shown in Fig. 8, we were also able to detect apoptosis induction in RajiH cells in the presence of both bsAb and PMN. According to our previously described results in breast cancer cells (7) and our current experiments with lymphoma cells (Fig. 8), we suggest that ADCC induced by PMN involves the induction of apoptosis in the target cells, which is supported by our current finding that the inhibition of caspases in target cells decreases HER-2/neu-directed cytotoxicity. However, cytotoxicity targeting HER-2/neu was not completely blocked by a broad spectrum caspase inhibitor, which suggests more than one pathway leading to PMN-mediated cytotoxicity against RajiH. The observed caspase-independent lymphoma cell death targeting HLA class II is in accordance with previous data (46) and suggests an important role for alternative apoptosis pathways. Caspase-independent induction of apoptosis involving NF-κB inhibition or the mitochondrial flavoprotein apoptosis-inducing factor has recently been described (47, 48). Therefore, we suppose the involvement of caspase-independent death pathways, although we cannot completely exclude the possibility that the induction of necrosis might also play a role in 51Cr release assays.

Chemical structure and lipophilic character enable DiI and DiO to insert into the lipid bilayers of the plasma membrane of intact cells where they display a free lateral diffusion. In general, the membrane dyes do not leak and move from cell to cell (34). Thus, the observed membrane dye exchange may represent a mutual transfer of membrane fragments between PMN and tumor cells, similar to trogocytosis. Trogocytosis was initially described in T cells, NK cells, and APC. It is induced after the activation of ITAM-containing surface molecules like the BCR or TCR (49). Furthermore, the temperature dependence of dye exchange indicates that membrane exchange is an active and energy-dependent process. Thus, our observation of membrane lipid exchange during neutrophil-mediated, Ab-dependent tumor cell lysis may be closely related to trogocytosis.

Because successful ADCC has always been associated with an intimate membrane interaction of PMN and target cells and with a mutual exchange of membrane lipids, both processes are supposed to mechanistically contribute to PMN-mediated ADCC and apoptosis induction. In conclusion, we suggest that the mutual membrane transfer between PMN and tumor cell targets that occurs during the formation of the “cytotoxic synapse” is involved in the induction of programmed target cell death.

Acknowledgments
We gratefully acknowledge the excellent technical assistance of B. Bock, S. Moi, and D. Thierschmidt. We gratefully thank Prof. J. G. J. van de Winkel for generously providing valuable reagents and Prof. G. H. Fey for useful discussions.

Disclosures
The authors have no financial conflict of interest.

References
1. Stadick, H., B. Stockmeyer, R. Kuhn, K. M. Schrott, J. R. Kalden, M. J. Glennie, J. G. van de Winkel, M. Gramatzki, T. Valerius, and D. Elsasser. 2002. Epidermal growth factor receptor and g250: useful target antigens for antibody mediated cellular cytotoxicity against renal cell carcinoma? J. Urol. 167: 707–712.
2. Stockmeyer, B., T. Valerius, R. Repp, I. A. Heijnen, H. J. Buhring, Y. M. Deo, J. R. Kalden, M. Gramatzki, and J. G. van de Winkel. 1997. Preclinical studies with FcγR bisppecific antibodies and granulocyte colony-stimulating factor-primed neutrophils as effector cells against HER-2/neu overexpressing breast cancer. Cancer Res. 57: 696–701.
3. Wurfein, D., M. Dechant, B. Stockmeyer, A. L. Tutt, P. Hu, R. Repp, J. R. Kalden, J. G. van de Winkel, A. L. Epstein, T. Valerius, et al. 1998. Evaluating antibodies for their capacity to induce cell-mediated lysis of malignant B cells. Cancer Res. 58: 3051–3058.
4. Di Carlo, E., G. Forini, P. Lollini, M. P. Colombo, A. Modesti, and P. Musiani. 2001. The intriguing role of polymorphonuclear neutrophils in antimicrobial reactions. Blood 97: 339–345.
5. Liu, M. Z., M. A. Teitel, and G. J. Schiller. 2005. The evolution of antibodies into versatile tumor-targeting agents. Clin. Cancer Res. 11: 129–138.
6. Glennie, M. J., and J. G. van de Winkel. 2003. Renaissance of cancer therapeutic antibodies. Drug Discov. Today 8: 503–510.
7. Stockmeyer, B., T. Beyer, W. Neuhuber, R. Repp, J. R. Kalden, T. Valerius, and M. Herrmann. 2003. Polymorphonuclear granulocytes induce antibody-dependent apoptosis in human breast cancer cells. J. Immunol. 171: 5124–5129.
8. Ottomeello, L., P. Morone, P. Dapino, and F. Dallegri. 1996. Monoclonal Lym-1 antibody-dependent lysis of B-lymphoblastoid tumor targets by human complement and cytoxin-exposed mononuclear and neutrophilic polymorphonuclear leucocytes. Blood 87: 5171–5178.
9. Stockmeyer, B., D. Elsasser, M. Dechant, R. Repp, M. Gramatzki, M. J. Glennie, J. G. van de Winkel, and T. Valerius. 2001. Mechanisms of GC-CSR- or GM-CSF-stimulated tumor cell killing by Fc receptor-directed bispecific antibodies. J. Immunol. Methods 248: 103–111.

10. Otten, M. A., and M. van Egmond. 2004. The Fc receptor for IgA (FcγRIIA) (CD16) as a novel trigger molecule for bispecific antibody therapy. Blood 99: 4485–4482.

11. Wagner, C., C. Iking-Konert, B. Denefleh, S. Stegmaier, F. Hug, and G. M. Hansch. 2004: Granzyme B and perforin: constitutive expression in human polymorphonuclear neutrophils. J. Immunol. 20: 463–469.

12. Ravelich, J. V. 1997. Fc receptors. Curr. Opin. Immunol. 9: 121–125.

13. Clynes, R., A. L. Towers, L. G. Presta, and J. V. Ravelich. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat. Med. 6: 446–446.

14. Cartron, G., L. Dacheux, G. Salles, P. Solal-Celigny, P. Bardos, P. Colombat, and H. Watier. 2002. Therapeutic activity of humanized anti-CD20 monoclonal antibod and polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. J. Immunol. 164: 1047–1051.

15. Treon, S. S., and C. J. Froelich. 2004. Human neutrophils lack granzyme A, granzyme B, and perforin: no doubt about it. J. Immunol. 171: 2517–2523.

16. Ottenello, L., A. L. Epstein, M. Mancini, G. Tortolina, P. Dapino, and F. Dallegri. 2005. Neutrophils express the high affinity receptor for IgG (FcγRI, CD64) after in vivo reapplication of recombinant human granulocyte colony-stimulating factor. Blood 85: 885–889.

17. Stockmeyer, B., M. Dechant, M. van Egmond, A. L. Tutt, K. Sundarapandiyan, R. F. Graziano, R. Repp, J. R. Kalden, M. Gramatzki, and M. J. Glennie et al. 2000. Triggering Fcα-receptor I (CD89) recruits neutrophils as effectors for cell CD20-directed antibody therapy. J. Immunol. 165: 5954–5961.

18. Cohen, J. J. 1993. Apoptosis. Immunol. Today 14: 126–124.

19. Metkar, S. S., and C. J. Froelich. 2004. Human neutrophils lack granzyme A, granzyme B, and perforin. Blood 104: 905–906.

20. Kataoka, T., A. Yamada, M. Bando, T. Homma, K. Mizoue, and K. Nagai. 2000. FD-891, a structural analogue of concanamycin A that does not affect vascular acidification or perforin activation, yet potently prevents cytotoxic T lymphocyte-mediated cytolysis by the blockage of conjugate formation. Immunology 100: 170–177.

21. Dechant, M., G. Vidarsson, B. Stockmeyer, R. Repp, M. J. Glennie, M. Gramatzki, J. G. van De Winkel, and T. Valerius. 2002. Chimeric IgA antibodies against HLA class II effectively trigger lymphoma cell killing. Blood 100: 4574–4580.

22. Otten, M. A., and M. van Egmond. 2004. The Fc receptor for IgA (FcγRIIA) (CD16) as a novel trigger molecule for bispecific antibody therapy. Blood 99: 4485–4482.

23. Cohen, J. J. 1993. Apoptosis. Immunol. Today 14: 126–124.

24. Stockmeyer, B., M. Dechant, M. van Egmond, A. L. Tutt, K. Sundarapandiyan, R. F. Graziano, R. Repp, J. R. Kalden, M. Gramatzki, and M. J. Glennie et al. 2000. Triggering Fcα-receptor I (CD89) recruits neutrophils as effectors for cell CD20-directed antibody therapy. J. Immunol. 165: 5954–5961.

25. Otten, M. A., and M. van Egmond. 2004. The Fc receptor for IgA (FcγRIIA, CD89). Immunol. Lett. 92: 23–21.

26. Cohen, J. J. 1993. Apoptosis. Immunol. Today 14: 126–124.

27. Stockmeyer, B., M. Dechant, M. van Egmond, A. L. Tutt, K. Sundarapandiyan, R. F. Graziano, R. Repp, J. R. Kalden, M. Gramatzki, and M. J. Glennie et al. 2000. Triggering Fcα-receptor I (CD89) recruits neutrophils as effectors for cell CD20-directed antibody therapy. J. Immunol. 165: 5954–5961.

28. Otten, M. A., and M. van Egmond. 2004. The Fc receptor for IgA (FcγRIIA, CD89). Immunol. Lett. 92: 23–21.

29. Cohen, J. J. 1993. Apoptosis. Immunol. Today 14: 126–124.

30. Stockmeyer, B., M. Dechant, R. Repp, M. J. Glennie, M. Gramatzki, M. J. Glennie, J. G. van de Winkel, and T. Valerius. 2001. Mechanisms of GC-CSR- or GM-CSF-stimulated tumor cell killing by Fc receptor-directed bispecific antibodies. J. Immunol. Methods 248: 103–111.

31. Metkar, S. S., and C. J. Froelich. 2004. Human neutrophils lack granzyme A, granzyme B, and perforin. Blood 104: 905–906.

32. Kataoka, T., A. Yamada, M. Bando, T. Homma, K. Mizoue, and K. Nagai. 2000. FD-891, a structural analogue of concanamycin A that does not affect vascular acidification or perforin activation, yet potently prevents cytotoxic T lymphocyte-mediated cytolysis by the blockage of conjugate formation. Immunology 100: 170–177.

33. Dechant, M., G. Vidarsson, B. Stockmeyer, R. Repp, M. J. Glennie, M. Gramatzki, J. G. van De Winkel, and T. Valerius. 2002. Chimeric IgA antibodies against HLA class II effectively trigger lymphoma cell killing. Blood 100: 4574–4580.