

Selective Binding of a Monoclonal Antibody to *Aspergillus Niger* Glucose Oxidase by Formaldehyde Fixed Human Polymorphonuclear Leukocytes

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Background: Many of the procedures used in handling neutrophils may affect the expression of surface antigens, and hence their quantitation by flow cytometry.

Methods: Because the enzyme glucose oxidase of *Aspergillus niger* is absent in human tissues, an IgM against it (mAb GO) was used as negative control in a study involving the normal expression of neutrophil specific BH2-Ag in different age groups.

Results: When peripheral blood leukocytes (PBL) were freshly prepared, processed and stained with FITC-mAb GO without fixation or when the cells were stained with FITC-mAb GO prior to fixation with 2% formaldehyde, both median fluorescent intensity (MFI) and per cent of positively stained polymorphonuclear leukocytes (PMN) were similar to that obtained with a background sample without any antibody. However, when PBL were fixed after isolation with different concentrations of formalde-

hyde and for varying durations, MFI and per cent of positively stained PMN but not of monocytes or lymphocytes with FITC-mAb GO increased in a time and concentration dependent manner. Saturation was achieved at a finite concentration of the antibody. In a competition assay unlabelled mAb GO reduced binding of FITC-mAb GO to PMN by 79% and 95% at concentrations 100 and 200 times that of FITC labelled antibody, respectively.

Conclusions: These observations strongly suggest that formaldehyde fixation causes the expression or accessibility of an epitope on PMN that is specifically recognized by the mAb GO. Cytometry 39:260–265, 2000.

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Key terms: formaldehyde; glucose oxidase; monoclonal antibody; polymorphonuclear leukocytes

Determination of neutrophil surface antigens by flow cytometry poses many difficulties. Many of the procedures used in preparing leukocytes for flow cytometry, including dextran sedimentation, mechanical manipulation, temperature changes, incubation with antibodies and fixation with formaldehyde (1–4) have been shown to effect the expression of these surface antigens.

In a longitudinal study to determine the expression of neutrophil specific BH2-Ag (5) in a population from premature babies to healthy adolescents and adults (manuscript in preparation), a mouse monoclonal (mAb) IgM prepared against glucose oxidase of *Aspergillus Niger* (mAb GO) was used as the negative control. According to the manufacturer, the enzyme is neither present nor inducible in mammalian tissues. In this report, we describe the unexpected effect of formaldehyde fixation of peripheral blood leukocytes (PBL) on the selective binding of the mAb GO to polymorphonuclear leukocytes (PMN).

MATERIALS AND METHODS

mAb BH2-C6

mAb BH2-C6 (an IgM with K light chain) specific for the BH2-Ag was prepared and purified from ascites in BALB/C mice as previously described (5). Purified mAb BH2-C6 was then coupled to fluorescein isothiocyanate (FITC; Sigma Chemical Co., St Louis, MO) using a previously described procedure (6) with slight modifications (7): Briefly, mAb BH2-C6 (8.1 mg/mL) in phosphate buffered saline (PBS) containing 0.02 % NaN₃ (PBS-NaN₃) was transferred to Na₂CO₃ solution (1.1M, pH:9.0) and concentrated to 11 mg/mL by ultrafiltration (Amicon Corp, Lexington, MA). FITC (1 mg/mL) in dimethylsulfoxide

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(DMSO) (Sigma) was very slowly added in the dark to the mAb BH2-C6-containing solution to a final concentration of 50 μg FITC per mg of protein. The mixture was then gently agitated in the dark. After 9h of agitation at 6°C, NH_4Cl (Fisher Scientific Co., Fair Lawn, NJ) was added to a final concentration of 50 mM. Free FITC was removed from the reaction by gel filtration on Sephadex G-25 (medium) in PBS-NaN_3 . The final concentration of FITC mAb BH2-C6 was 2 $\mu\text{g}/\mu\text{L}$.

mAb GO

FITC-mAb GO was purchased from DAKO Corp (Carpinteria, CA) (code no. X 934) at 75 $\mu\text{g}/\text{mL}$. Unlabelled (without FITC) mAb GO (code no. X 942) at 100 $\mu\text{g}/\text{mL}$ was also purchased from the same source.

Preparation of PBL

Cord blood from newborns and venous blood from healthy children and adults was collected into ethylenediaminetetraacetic acid (EDTA) tubes and processed within 2h of collection. All blood collections were done during routine deliveries and examinations in accordance with a protocol approved by the Institutional Review Board, and informed consent was obtained from the parents of the children. Erythrocytes (RBC) were first sedimented in 1.5% dextran (Pharmacia AB, Uppsala, Sweden) for 30 min at room temperature (RT). Following centrifugation of the buffy coat for 10 min at 250g, the remaining RBC were lysed in 5 mL of 0.2% ice cold saline for exactly 90 sec when the salt concentration was balanced by addition of an equal volume of 1.6% saline. After centrifugation into a pellet the PBL were resuspended and counted in PBS.

mAb-Staining

For each mAb staining procedure 1×10^6 PBL were used. Cells, freshly isolated or fixed (see below) were first suspended in 100 μL of PBS-NaN_3 . FITC-mAb BH2-C6 or FITC-mAb GO was added at a previously determined concentration of 2 $\mu\text{g}/10^6$ cells and the mixture was incubated for 45 min in ice. To assure appropriate mixing, tubes were gently agitated every 15 min. To separate nonreacted mAb from the cell-bound mAb, the mixture was layered carefully on top of 1 mL of 100% fetal bovine serum (FBS) (Gibco Laboratories, Chagrin Falls, OH) and the PBL passed through the FBS by centrifugation for 5 min at 150g, 4°C. After removal of the FBS-supernatant the PBL were washed 2 times in PBS and either directly analyzed by flow cytometry or subjected to fixation and analysis by flow cytometry.

Cell Fixation

Formaldehyde (Fisher Scientific) at 0.2 and 2% in PBS was used as fixative. Cell fixation was done in the following two ways: Either freshly isolated cells were first reacted with the respective FITC-mAb, resuspended and then fixed with 2% formaldehyde or PBL were first fixed in 0.2 or 2% formaldehyde for various periods of time,

washed and then reacted with the respective FITC-mAb as described in Results.

Binding and Competition Assay

Freshly obtained white blood cells were fixed with 2% formaldehyde-PBS for 2 h, extensively washed and 10^6 cells/tube were distributed into a series of Eppendorf tubes containing 0.1 mL PBS-BSA. Amounts of FITC-mAb GO were then added to each tube to generate a dilution curve from 0.01 to 40 $\mu\text{g}/10^6$ cells. After 45 min on ice the cells were passed through FBS, processed for cytofluorography and analyzed with respect to fluorescence as described below.

For the competition assay, the fixed cells distributed into a series of Eppendorf tubes were first incubated in PBS-BSA containing unlabelled mAb GO at concentrations from 0.05 to 100 μg each/ 10^6 cells. After 15 min on ice, a standard amount of 0.5 μg of FITC-mAb GO was added into each tube and the incubation was allowed to continue for another 45 min on ice when the cells were processed for cytofluorography as above. A control sample was processed identically but without any unlabelled mAb GO. Efficiency of competition was assessed by measuring the fluorescence of bound antibody as defined below.

Flow Cytometry

Flow cytometry was performed using a FACS SCAN analyzer (Becton Dickinson, San Jose, CA) equipped with an Argon laser wave length 488 A and connected to Hewlett Packard computer using the LYSYS II program by BD. The PMN, lymphocytes and monocytes in the PBL preparations were identified for selective FACS analysis by their characteristic forward and right angle light scattering (Fig. 1A). Fluorescence of the selected PMN population was then determined on an average of 10^4 cells using an arbitrary log scale (10^0 - 10^4) to define fluorescence intensity. As will become evident from the results, the background fluorescence with a "non-specific" FITC-mAb IgM could only be established using unfixed PMN or PMN stained before fixation; background fluorescence for PBL stained after fixation could not be obtained in this way. Thus, background fluorescence was evaluated, in addition, on PMN fixed and processed without FITC-mAb to measure any probable autofluorescence. However, no significant change was seen in the fluorescence of unstained fixed versus unfixed PMN (Fig. 1B). Analysis was done concerning the mean fluorescence intensity (MFI) for each PMN sample and the percent of cells stained. No background value corrections were done on the representative samples shown in results for each of the conditions examined.

RESULTS

The scattergram of a typical analysis of unfixed cells with the definition of the cell populations and the FACS analysis of PMN is shown in Figure 1. The distinctly different fluorescence readings for unstained PMN (Fig. 1B) and PMN stained with FITC-mAb GO (Fig. 1C) from those

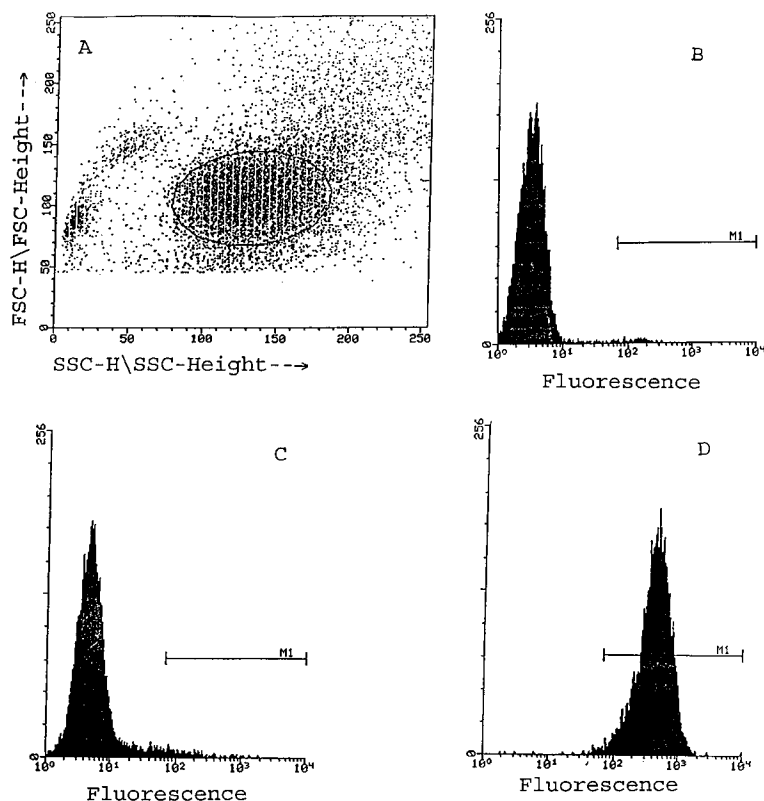


FIG. 1. Dotplot of forward light scatter (FSC) versus side scatter (SSC) (A) and histograms of fluorescence distribution of fresh and unfixed PMN after reaction with no mAb (B), with FITC-mAb GO (C) and FITC-mAb BH2C6 (D).

for FITC-mAb BH2C6 (Fig. 1D) is clearly evident from this typical example. When freshly isolated and unfixed PBL were stained with FITC-mAb BH2-C6 and the PMN population analyzed, a single peak of strongly fluorescent PMN (MFI 506 ± 36) was seen (Fig. 1D) covering nearly 99% of all cells within the gate set for PMN (Fig. 1A). In contrast, the MFI (11.5 ± 1.5) for PMN reacted with FITC-mAb GO was 2 logs below that of FITC-mAb BH2-C6 and indistinguishable from the MFI (6.5 ± 0.5) for unstained PMN (Table 1). FITC-mAb BH2-C6 stained $98.9 \pm 0.08\%$ of all unfixed PMN.

If fresh PBL were first stained with either one of the FITC-mAb, fixed overnight with 2% formaldehyde, extensively washed, resuspended to 10^6 cells/mL PBS and analyzed as above, the histograms were indistinguishable from those obtained with unfixed cells. In addition, the MFI and per cent of PMN stained with FITC-mAb BH2-C6 without or after subsequent fixation were closely similar while virtually no staining was seen using FITC-mAb GO (Table 1).

The results were quite different when the PBL were first fixed overnight in 2% formaldehyde in PBS before being washed extensively and suspended in PBS-BSA before being treated with FITC-mAb, and analyzed by FACS. As shown in Table 1, line 3, FITC-mAb GO not only reacted strongly with the PMN (MFI 640 ± 34) but also with $>99\%$ of PMN. The results further show that the reactivity of FITC-mAb GO with prefixed PMN is virtually the same as that seen with the FITC-mAb BH2-C6 (Table 1).

Recently (3), it was observed that shorter fixation times combined with a lower formaldehyde concentration significantly reduced nonspecific fluorescence of PBL as seen in FACS analysis. In the next experiment, therefore, we reduced the fixation time from 14 hours to 60, 10 and 4 min using 2% formaldehyde-PBS. Samples from the same cell preparations were treated in parallel with 0.2% formaldehyde-PBS for the same time periods prior to exposure to FITC-mAb.

As expected, the exposure of PBL to either concentration of formaldehyde for the given time periods exerts little effect on the amount of FITC-mAb BH2C6 bound by the PMN as expressed by the MFI (Table 2) or on the per cent of PMN stained with FITC-mAb BH2-C6 (Table 3). In contrast, fixation of PBL for 60 min with only 0.2% formaldehyde resulted in a >10 fold increase not only in the MFI but also in per cent of PMN stained with FITC-mAb GO as compared to cells stained without fixation at all or after 4 and 10 min of fixation. When the treatment in 0.2% formaldehyde-PBS was extended to 14 h, the number of PMN stained (Table 3) and the MFI levels (Table 2) reached were very similar to those seen with FITC-mAb BH2-C6. Binding of FITC-mAb GO to PMN was even more evident when the PBL were fixed with 2% formaldehyde. Already after 4 min fixation, the fluorescence intensity was equivalent to that of FITC-mAb BH2-C6 bound specifically by $>99\%$ of the PMN (MFI of 481 and 453, respectively). However, only 52.3% of the PMN fell within the gating area set on the BH2-Ag⁺ population. Prolonging the

Table 1
Effect of Different Sequences of Treatment on the Staining of PMN by FITC-mAbs

Processing of cells	FITC-labelled mAb used					
	None	–GO	–BH2C6	None	–GO	–BH2C6
	MFI \pm SEM ^a	MFI \pm SEM	MFI \pm SEM	% ^b	%	%
Staining—FACS	6.5 \pm 1.5	11.5 \pm 1.5	506 \pm 36	0.5	1.8	99.0
Staining—FA ^c —FACS	8.5 \pm 0.5	12.5 \pm 2.5	547 \pm 126	1.9	2.0	99.0
FA ^d —Staining—FACS	6.4 \pm 1.1	640 \pm 34	671 \pm 62	0.3	99.56	100.0

^aMedian Fluorescence Intensity \pm Standard Error of the Mean (n = 5–6 samples each).

^bPercent of total PMN stained.

^cStaining with FITC-mAb followed by fixation with 2% paraformaldehyde in PBS overnight, washed and resuspended in PBS, resuspended to 10⁶ cells/mL and cytofluorographed.

^d2% formaldehyde overnight, washed 2 \times with PBS, 1 \times with PBS-1%BSA, reacted with FITC-mAb in PBS-1% BSA, washed and resuspended in PBS to 10⁶ cells/mL and cytofluorographed.

Table 2
*The Effect of Formaldehyde Concentration and Duration of Fixation on the MFI of PMN Treated with FITC mAb**

FITC mAb	Unfixed	0.2% formaldehyde				2% formaldehyde			
		4 ^a	10	60	14h	4 ^a	10	60	14h
	MFI	MFI	MFI	MFI	MFI	MFI	MFI	MFI	MFI
None	3	4	2	3	2	5	7	4	5
GO	13	17	20	189	416	481	930	496	777
BH2C6	470	673	523	478	719	453	562	559	770

*A representative example of 3 experiments is given.

^aExposure to fixative in min at 4°C.

Table 3
*The Effect of Formaldehyde Concentration and Duration of Fixation on the Percentage of Total PMN Reacting with FITC mAb**

FITC mAb	Formaldehyde concentration	Fixation time			
		4 min	10 min	60 min	14h
		% ^a	%	%	%
BH2C6	0.2%	99.4	99.4	98.8	99.3
GO	0.2%	2.5	3.6	30.5	81.3
GO	2%	52.3	69.2	42.8	99.5

*A representative example of 3 experiments is given.

^aPercent of total PMN stained.

fixation time to 60 min did not significantly change the MFI nor the number of PMN stained with FITC-mAb GO. An extension of the fixation time in 2% formaldehyde to 14 h, on the other hand, resulted in more than doubling from 43% to 99% of the PMN that bound FITC-mAb GO with an MFI of 777, values usually observed only with a PMN-specific antibody such as mAb BH2-C6 (99-100% PMN stained, MFI 770). The question arose whether the binding of FITC-mAb GO to formaldehyde fixed but not to unfixed PMN occurs due to an event unique to PMN. Consequently, reaction of FITC-mAb GO to unfixed and fixed mononuclear cells was examined. This was done by extraction of the data for lymphocytes and monocytes from the same FACS analyses that provided the results for PMN. As can be seen from Table 4 virtually no binding was observed of FITC-mAb GO nor of FITC-mAb BH2-C6 to lymphocytes and monocytes fixed for up to 14h with 2% formaldehyde.

In order to prevent any nonspecific binding of the mAbs all staining procedures were carried out in PBS containing 1% BSA as detailed in Material and Methods. In addition, after the staining procedure, PBL were passed through 100% FBS. Because both of these approaches failed to prevent the binding of FITC-mAb GO, two additional processing steps were added in an attempt to quench non-specific binding sites. First, the cells were incubated in PBS supplemented with 10% FBS for 15 min prior to staining. Secondly, in a separate experiment, PBL were first incubated in unlabelled purified mouse myeloma IgM (1 μ g/ μ L) (Zymed Lab Inc., San Francisco, CA) for 15 minutes before staining with FITC mAb GO. Both of these additional approaches, however, failed to prevent the binding of FITC-mAb GO to PMN (data not shown).

To ascertain that the binding of FITC-mAb GO occurs in a specific manner, concentration-dependent binding was determined. Furthermore, a competitive inhibition assay

Table 4
MFI and Per Cent of Monocytes and Lymphocytes Stained with FITC mAb*

Cells ^a	FITC-labelled mAb					
	None	-GO	-BH2C6	None	-GO	-BH2C6
	MFI	MFI	MFI	% ^b	%	%
Lymphocytes	2.2	10.7	4.5	0.0	0.3	0.4
Monocytes	4.3	32.7	10.0	0.0	1.9	1.2

*A representative example of 3 experiments is given.

^aCells were fixed for 14h in 2% formaldehyde-PBS, washed, stained with FITC mAbs 2 $\mu\text{g}/10^6$ cells and processed for FACS analysis.

^bPercent of total lymphocytes or monocytes stained.

was designed to establish to what extent, if any, the preincubation of PBL with unlabelled mAb GO would decrease the MFI of FITC-mAb GO. In both assays PBL fixed for 2 h in 2% formaldehyde-PBS were used. For the binding curve, the cells were extensively washed before they were resuspended in PBS - BSA and incubated for 45 min on ice with increasing concentrations of FITC-mAb GO. FACS analysis of the PMN population shows that after little retention at low concentrations (0.01 - 0.1 $\mu\text{g}/10^6$ cells) FITC-mAb GO binds to PMN in increasing amounts up to $\approx 8 \mu\text{g}/10^6$ cells when the slope of the binding curve begins to decrease (Fig. 2A) and binding begins to plateau indicating saturation. Since this experiment further showed that 0.5 μg FITC-mAb GO stains >99% of all PMN with an MFI of ≈ 300 , this concentration was chosen as the FITC-mAb reference value in the competition assay.

In this assay, PBL were fixed as described above, then preincubated with increasing concentrations of unlabelled mAb GO followed by incubation with the standard amount of 0.5 μg FITC-mAb GO/ 10^6 cells. It can be clearly seen from Figure 2B that unlabelled mAb GO at 25, 50 and 100 $\mu\text{g}/10^6$ cells (i.e. 50, 100 and 200 times the concentration of FITC-mAb GO) effectively reduces binding of FITC-mAb GO to PMN by 71 %, 79 % and 95 %, respectively. In fact, binding inhibition of the FITC-mAb GO begins already when only 3 - 6 μg of unlabelled mAb GO, i.e. 6 to 12 times the amount of FITC-mAb GO, are present during the incubation. Together, saturation binding of FITC-mAb GO and the result of the competition assay strongly indicate that FITC-labeled and unlabeled mAb GO compete for a limited number of sites with the identical antigenic epitope.

DISCUSSION

It is well known that physicochemical and mechanical conditions used during cell preparation can significantly affect the expression of neutrophil surface antigens (1-4). Macey et al (1) have shown that prolonged fixation with 0.2% formaldehyde causes increases in the MFI of PMN that had been reacted with IgG₁ control mAbs. An extension in the fixation time from 4 to 55 min resulted in an 11 fold increase in the MFI to 43.3 for one of their IgG₁ antibodies. The authors have suggested that increases in membrane permeability which they showed to occur with prolonged formaldehyde fixation, may expose cytoplasmic structures (antigens?) to the mAb causing the in-

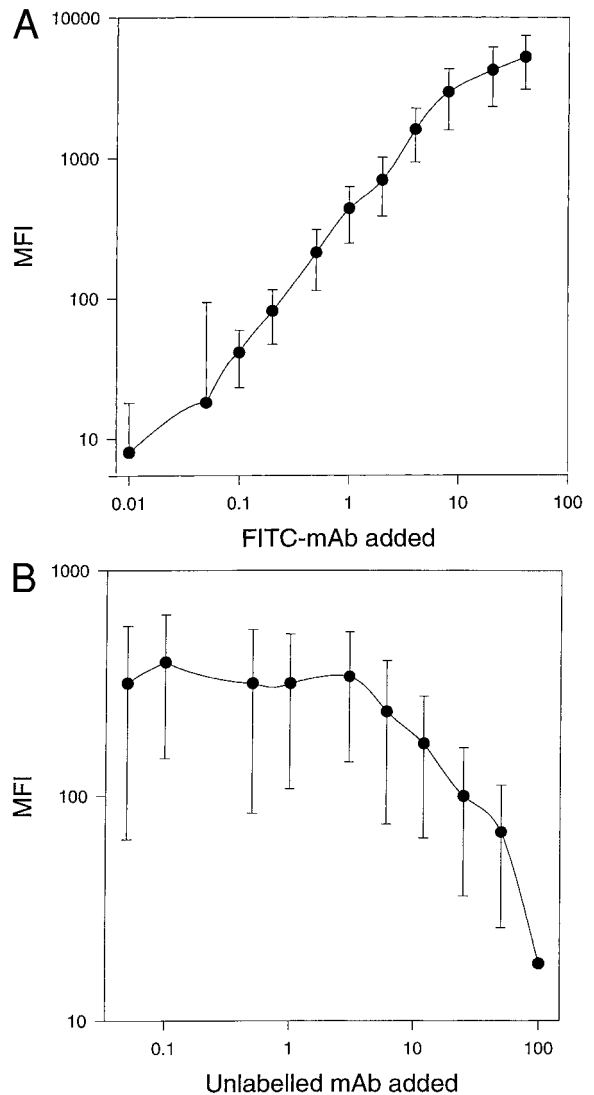


FIG. 2. A: Binding of FITC-mAb GO to formaldehyde fixed PMN. FITC-mAb GO is expressed as the log of the amount of antibody in $\mu\text{g}/10^6$ cells and fluorescence as the log of MFI \pm SD. B: Competition assay. FITC-mAb GO is added at a standard concentration of 0.5 μg to each sample. Unlabelled mAb GO added is expressed as log of the amount of antibody in $\mu\text{g}/10^6$ cells and fluorescence as the log of MFI \pm SD.

creased retention of the FITC-mAb and, consequently, the MFI. They also suggested that free fluorochrome, if present in the FITC labelled antibody solution, might be a

component reactive with and, thus, be retained by the intracellular structures (1).

Interaction with and binding of mAb BH2-C6 to the BH2-Ag that has been shown to be exquisitely expressed by human neutrophils but not other blood cells (5), is unaffected by formaldehyde fixation. FITC-mAb BH2-C6 reacts equally well with 98–100% of unfixed neutrophils and neutrophils fixed with 0.2 and 2% formaldehyde irrespective of the length of fixation. FITC-mAb BH2-C6 binding remains high with an MFI of 400–770 in all the conditions examined. These findings correspond well with and extend previous observations (5, 7) that demonstrated binding of mAb BH2-C6 to live cells and cells fixed for various time periods with 2–4 % paraformaldehyde or 2% glutaraldehyde (5,7).

In contrast, FITC-mAb GO that gives an MFI reading equal to background on freshly prepared and unfixed PMN, binds in significant amounts to PMN fixed for only 4 min when fixation is performed with 2% formaldehyde. When PMN are processed with 0.2% formaldehyde, binding of FITC-mAb GO is insignificant and comparable to background when the fixation period lasts only 4 to 10 min. After 60 min of treatment with 0.2% formaldehyde, however, the MFI is 11 fold higher for FITC-mAb GO binding than that measured after only 4 min of fixation, an increase similar to that observed by Macey et al (1). Even though these authors' ability to stain for vimentin in formaldehyde fixed cells using unlabeled whole anti-vimentin IgG followed by rabbit FITC-F(ab')₂ against mouse Ig suggests a state of increased permeability of the cells plasma membrane for IgG, it is highly unlikely that such change in membrane permeability of the PMN could have resulted in the increased binding of FITC-mAb GO.

First, both antibodies used in this study are of the mouse IgM isotype and, thus, are much larger (6.5 times just by molecular weight) than IgG antibodies. Being such large molecules IgM antibodies are unlikely to penetrate effectively the plasma membrane without prior permeabilization by detergents such as Triton X-100.

Second, had the FITC-mAb BH2-C6 in fact penetrated the plasma membrane of the PMN, a very significant elevation in the MFI of BH2C6-stained cells should have been observed as compared to the intact i.e. live cells, since >5 times more BH2-Ag is found to be present intracellularly than on the neutrophil plasma membrane (unpublished experiments). The absence of any increase in the fluorescence of fixed versus the unfixed PMN by FITC-BH2-C6, a mouse IgM, is a strong indication that no cytoplasmic staining had occurred. This observation strongly argues against the possibility that mAb GO, also a mouse IgM and used under virtually identical conditions would have been able to penetrate the fixed plasma membrane.

Third, even if the antibody had penetrated the membrane it would not be expected to give any positive reading, since the antigen, glucose oxidase of *aspergillus niger*, is according to the best of our knowledge and the manufacturer's information (Dako, verbal communication) neither present nor inducible in mammalian cells. A search via Swiss-Protein information system for a structur-

ally similar protein in man produced the only homology within a stretch of 26 amino acids (42% identity) of human amine oxidase (total sequence 527 amino acids) (EC 1.4.3.4). While being also a member of the flavoprotein oxidoreductases, amine oxidase is widely distributed in tissues and cells where it has been localized to the mitochondrial outer membrane (8,9). Taking into consideration the points made above, it is highly unlikely that binding of FITC-mAb GO to PMN occurs via amine oxidase. In fact, this interaction can be excluded by the finding that FITC-mAb GO stained only PMN but not lymphocytes and monocytes.

The most likely explanation, therefore, for this phenomenon is that the formaldehyde fixation of PMN causes a novel epitope to be expressed and/or become accessible to the FITC-mAb GO on the cells' plasma membrane. The shape of the binding curve (Fig. 2A) of FITC-mAb GO to fixed PMN which appears to reach a plateau, indicates that there are a finite number of antigenic sites on the plasma membrane of the fixed cells. It is also possible that the apparent saturation of mAb GO-binding sites may represent a quenching phenomenon observed at high antibody density on the surface membrane of the fixed PMN. Irrespective of the underlying mechanism that restricts further binding of mAb GO, the effective competition of FITC-mAb GO binding by its unlabeled counterpart (Fig. 2B) but not by BSA alone, nor by concentrations of FBS up to 100%, and, most importantly, by an excessive amount of mouse myeloma IgM, strongly suggests the existence of a unique mAb GO-specific epitope on the plasma membrane of formaldehyde-fixed PMN. Present investigations with respect to the isolation of this antigen from formaldehyde-fixed PMN will allow to delineate the structure of this epitope.

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