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ACTIVATION OF HUMAN PHAGOCYTES THROUGH CARBOHYDRATE ANTIGENS (CD15, SIALYL-CD15, CDw17, AND CDw65)[1]

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The leukocyte carbohydrate (CHO) Ag CD15, sialyl-CD15, and CDw65 have recently been found to function as ligands for CD62 and ELAM-1 cell adhesion molecules on platelets and endothelium, respectively. Cell adhesion ligands also may act as receptors capable of signal transduction. We therefore investigated the possibility that these CHO Ag and CDw17, a glycolipid Ag whose expression is regulated by leukocyte activation, may have receptor-like characteristics. The effects of antibody cross-linking of CHO Ag on phagocyte activation were measured by using flow cytometry and fluorescent indicators for cytoplasmic calcium ions, oxidative burst, and the granule-associated proteins CD11b and CD67. Cross-linking of CD15, sialyl-CD15, CDw65, or CDw17 induced a moderate release of calcium ions into the cytoplasm of granulocytes, a strong activation of oxidative burst, and a low up-regulation of CD11b and CD67 compared to the effects of treatment with 4 μM FMLP. The results suggest a role for CHO Ag in leukocyte signal transduction and support the view that these molecules are involved in phagocyte activation.

Human monocytes and granulocytes express large amounts of complex CHO[3] Ag on their cell surface (1–5). Recently, it has been shown that some of these CHO mediate adhesion of granulocytes to activated platelets and endothelium (6–12). The CD15 epitope (Gal^{b}1–4(Fuc^{a}1–3)GlcNAc^{b}1-R) (1) and CD15 (2) have been characterized as ligands for CD62 (GMP-140) present on activated platelets (6, 11), whereas s-CD15 and CDw65 (a ceramide-dodecasaccharide ^{a}1–3 fucosylated on the penultimate glCnAC) (5) have been shown to interact with the ELAM-1 molecule on activated endothelium (8, 12).

Several cell adhesion molecules serve a dual role, acting both as binding sites for molecules on extracellular matrix or other cells, and as receptors capable of transmembrane signal transduction. In experiments where the physiologic multivalent engagement of adhesion molecules has been mimicked by antibody cross-linking, it has been shown that molecules such as CD2 (13), CD11a (14), CD11b, CD35 (15), and CD44 (16) can act as receptors and mediate cell activation. Although several reports have shown that mAb against CHO Ag may influence phagocyte functions (17–24), there is limited information about potential involvement of CHO Ag in transmembrane signaling in human monocytes and granulocytes.

The present study investigated the possibility that phagocyte CHO Ag may have receptor-like characteristics. Flow cytometry and fluorescent indicators were used to study the effects of antibody cross-linking of these molecules on cytoplasmic calcium levels, oxidative burst, and degranulation of leukocyte subsets. The antibodies used were specific for the carbohydrate epitopes CD15, s-CD15, CD43 (leukosialin) (25), CDw17 (lactosylceramide) (3), and CDw65, adhesion molecules including CD11b/CD18 (CR3) (26), CD11a (LFA1) (26), and CD44 (hyaluronic acid receptor) (27) and CD45 (protein tyrosine phosphatase) (28). Our results demonstrate that in the absence of other stimuli, cross-linking of cell-bound mAb to several CHO Ag induces cytoplasmic calcium fluxes, activation of the respiratory burst, and degranulation.

MATERIALS AND METHODS

Reagents. Dulbecco's PBS with or without calcium and magnesium (PBS-Ca or PBS, respectively) and FCS were purchased from Gibco (Paisley, Scotland, United Kingdom). FMLP, PMSF, and pepsin were from Sigma Chemicals Co. (St. Louis, MO). Dihydrorhodamine 123 and Fluo-3 were from Molecular Probes (Eugene, OR). FMLP (10 mM), Dihydrorhodamine 123 (10 mg/ml), Fluo-3 (1 mM), and PMSF (100 mM) were dissolved in dry DMSO (Merck, Darmstadt, Germany) and stored in aliquots at −70°C. The solvent concentrations were less than 0.5% in final dilutions. Paraformaldehyde, and all salts used in laboratory-made solutions, were analytical grade. Microwell plates (polystyrene, V-bottomed) were from Nunc (Copenhagen, Denmark).

Polyclonal secondary antibodies. Fluorochrome-conjugated and unlabeled GAM-M, GAM-HL, and goat anti-mouse IgG (Fc-specific) were generously provided by Jackson Immunoresearch (West Grove, PA). Unconjugated secondary antibodies were used as 1.2 mg/ml in PBS, whereas conjugated antibodies were used as 20 μg/ml in PBS-FCS and 0.1% sodium azide. The unconjugated secondary antibodies were found to contain >99% pure F(ab′)$_2$ fragments by HPLC analysis using a TSK 3000W column (Toyo Soda Manufacturing, Japan) (data not shown).

mAb. The mAb used in the study are listed in Tables I and II. All

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[3] Abbreviations used in this paper: CHO, carbohydrate; s-CD15, sialylated form of CD15; PBS-FCS, PBS with 2% FCS and 5 mM glucose; GAM-M, F(ab′)$_2$ fragments of goat antibodies to mouse IgM μ-chain specific; GAM-HL,F(ab′)$_2$ fragments of goat antibodies to mouse IgG heavy and light chains; PBS-FCS-Ca, PBS with calcium, magnesium, 2% FCS, and 5 mM glucose.

mAb were diluted in PBS-FCS with 0.1% sodium azide and used at concentrations giving the maximal fluorescent staining and minimal cell aggregation as determined by flow cytometry. The concentrations of the commercial mAb varied from 2 to 50 μg/ml, whereas ascites fluids were used at 1:100 to 1:200 dilutions.

Preparation of F(ab')$_2$ fragments of IgG mAb. IgG was purified on a protein A Sepharose 4 fast flow column (Pharmacia, Stockholm, Sweden), dialyzed against citrate buffer (pH 3.5 for IgG1, pH 4.3 for IgG2a), and incubated with pepsin (25 μg/ml) for 18 h at 37°C. The digestion was stopped by the addition of 1 mM PMSF, and the solution dialyzed against PBS before intact IgG was removed by protein A chromatography. The purity of F(ab')$_2$ fragments was demonstrated by measuring a >99% reduction in cell staining with phycoerythrin-conjugated antibodies to the Fc part of mouse IgG, as compared to the intact IgG mAb. All F(ab')$_2$ fragments prepared in this way stained cells with the same patterns as the corresponding intact IgG antibodies (data not shown).

Removal of contaminating IgG from IgM ascites fluids. Ascites fluid preparations were dialyzed against 20 mM phosphate buffer (pH 7.2) and passed over a protein G Superose HPLC column (Pharmacia). The IgG-depleted preparations were then dialyzed against PBS without calcium and magnesium.

Isolation of leukocytes. Venous blood from healthy laboratory personnel between 20 and 45 years old was drawn into Vacutainers containing ACD solution A (Becton Dickinson, Oxnard, CA) and diluted 1:10 in a solution containing 0.8% NH_4Cl, 0.08% $NaHCO_3$, and 0.08% EDTA (pH 6.8, 20°C) to lyse RBC. The leukocytes were then pelleted by centrifugation at 180 × *g* for 5 min and washed once in PBS-FCS.

Labeling of cells with mAb. For functional experiments, as well as for immunofluorescence measurements, leukocytes were labeled with mAb and washed before the addition of secondary antibodies. This labeling procedure was chosen to prevent the formation of immune complexes when the secondary antibodies were added, and to allow better control of the ratio of F(ab')$_2$ fragments of goat antibodies to mAb when the concentrations of the mAb were unknown. Leukocytes were transferred to microwell plates (2×10^5 to 8×10^5/well) and pelleted by centrifugation at 160 × *g* for 3 min. The supernatant was then discarded, and the cells resuspended by whirlmixing. Ten microliters of the mAb solutions were added to each pellet, and the plates kept at 20°C for 25 min with whirlmixing every 7 min. The cells were then washed once in PBS-FCS.

Immunofluorescence. Leukocytes were labeled with mAb as described above, washed once with PBS-FCS, and stained with 10 μl of fluorochrome-conjugated antibodies for 30 min on ice. The antibody-labeled cells were washed, fixed in PBS containing 1% paraformaldehyde, and analyzed by flow cytometry.

Measurement of fluctuations in intracellular calcium concentration. Leukocytes (10^7/ml) were incubated 25 min at 37°C in PBS-FCS containing 2 μM Fluo-3-acetoxymethylester (29), washed, and transferred to microwell plates. The cells were then labeled with mAb as described above, washed, whirlmixed, and resuspended in 200 μl of cold (4–6°C) PBS-FCS-Ca. The cells were then kept at 4–6°C and prewarmed to 37°C for 90 s before the flow-cytometric measurement. Samples could be kept at 4–6°C for at least 4 h without detectable changes in the calcium fluxes induced by any mAb, whereas storage at 20°C led to decreased responses to several mAb, including Mo1, VIM2, and DAKO-15 (data not shown).

During the flow-cytometric analyses, the sample temperature was kept at 37°C with the help of a laboratory-made flow cytometry sample chamber. Monocytes and granulocytes were distinguished by measurements of cellular light scatter, and their Fluo-3 fluorescence gated vs time to separate cytograms (30, 31). Basal cytoplasmic calcium levels were recorded for 10 to 20 s before 10 μl F(ab')$_2$ fragments of goat antibodies were added to cross-link cell-bound mAb. Fluctuations in cytoplasmic free calcium concentration were recognized as alterations in Fluo-3 fluorescence intensity over time (30, 31). The mean fluorescence intensity of the cells at any given time point was calculated by software from Cytomation, Inc. (Englewood, CO). In this mean curve, the maximal cellular Fluo-3 fluorescence was compared to mean prestimulated levels, and a doubling or more was considered indicative of a stimulus-induced cytoplasmic calcium flux. Fluo-3 is regarded as a good indicator for observing relative changes in cytoplasmic calcium levels because it has high sensitivity at both high and low calcium concentrations (29). Fluo-3 is less reliable as an indicator for absolute calcium levels (29). However, this study only measured relative changes in cytoplasmic calcium, and no attempts were made to determine absolute concentrations.

To determine the source of mobilized calcium ions, antibody-labeled cells were resuspended in either PBS-FCS-Ca or PBS-FCS. To chelate any calcium ions present in the PBS-FCS solution, 100 μl of the sample were mixed with 100 μl of PBS-FCS containing 1.0 mM EGTA less than 1 min before flow-cytometric analysis.

Measurement of oxidative burst. Cellular oxidative burst was quantified by using the fluorescent indicator, Dihydrorhodamine 123 (32). Dihydrorhodamine 123 is a nonfluorescent, cell membrane-permeable compound. During the oxidative burst, the intracellular Dihydrorhodamine 123 is irreversibly converted to the fluorescent compound, Rhodamine 123 (32, 33). This rhodamine is membrane-impermeable, and so accumulates in the cells. The cellular oxidative burst is measured as a function of cellular Rhodamine 123 fluorescence intensity (32). The specificity of this indicator for oxidative burst has been demonstrated in experiments with cells from patients with chronic granulomatous disease (33). After labeling with mAb at 20°C (see above), cells were washed once in PBS-FCS (20°C). Ten microliters of GAM-M, GAM-HL, FMLP (40 μM), or PBS were added to each pellet, and the cells resuspended on a whirlmixer before 100 μl PBS-FCS-Ca prewarmed to 37°C, containing 10 μg/ml Dihydrorhodamine 123 were added, and the cells incubated at 37°C. After 20 min of incubation, 100 μl of ice-cold PBS-FCS containing 0.02% paraformaldehyde were added, and the samples kept on ice until flow-cytometric measurement. The combination of low paraformaldehyde concentration and low temperature was used to prevent leakage of intracellular dye that occurred over time with higher temperatures and higher concentrations of fixative. Increases of twofold or greater in cellular Rhodamine 123 fluorescence relative to control were considered as significant stimulus-induced respiratory bursts.

Up-regulation of surface expression of granule-associated proteins. Activation-induced up-regulation of the granule-associated proteins CD11b and CD67 was determined by flow-cytometric measurement of immunofluorescence (34, 35). CD11b and CD67 are associated with the specific granules of granulocytes and are translocated to the plasma membrane during degranulation (34, 35). In addition, CD67 has been suggested to be associated with the secretory granules or latent alkaline phosphatase compartment (35, 36). For measurements of Ag up-regulation, the cells were activated in the same manner as for oxidative burst measurements (see above) except that Dihydrorhodamine 123 was excluded from the solutions. After 20 min of incubation at 37°C, the cells were cooled, washed, and incubated on ice for 20 min with PBS containing 10% normal mouse serum to block nonspecific binding sites for IgG antibodies. The cells were then washed, stained on ice with the FITC-conjugated mAb Bear1 (anti-CD11b) or 80H3 (anti-CD67) for 20 min, washed in ice-cold PBS-FCS, fixed in 1% paraformaldehyde, and measured by flow cytometry. The specificity of 80H3 is based on it recognizing a 100-kDa phosphatidyl-inositol linked protein, and on the ability of the G10F5 anti CD67 mAb to block 80H3 binding to granulocytes.

Priming of leukocytes with FMLP. Venous blood was collected into 3-ml heparin Vacutainers (Becton Dickinson). FMLP (2×10^{-7} M) or PBS was then added, and the tube incubated in a 37°C water bath for 20 min with constant agitation. The leukocytes were then isolated and labeled with mAb as described above.

Flow cytometry and data analysis. Calcium measurements were performed with a Coulter Epics V flow cytometer (Coulter Electronics, Luton, United Kingdom) interfaced to a CICERO PC-based data aquisition and analysis system (Cytomation). The excitation source was a 488-nm argon laser, and standard fluorescein filters were used for Fluo-3 detection. The kinetics and amplitudes of calcium responses were analyzed using specially designed software from Cytomation. The software draws a curve of the mean fluorescence intensity of the cells in a population during the time of measurement. Measurements of immunofluorescence and Rhodamine 123 fluorescence were performed with Coulter Profile II or Becton Dickinson FACSscan flow cytometers with standard filter setup. Data obtained from the Profile cytometer were processed with Verity Isocontour software from Verity Softwarehouse (Topsham, ME) for graphic presentation.

Statistical methods. Significance of difference was evaluated by paired Student's *t*-tests.

All experiments were performed at least three times and with leukocytes from at least three different persons.

RESULTS

Expression of CHO Ag on leukocyte subsets. Granulocytes expressed high levels of CHO Ag as determined by flow-cytometric measurement of staining with anti-CHO mAb (Table I). The mAb to CD15 and s-CD15 were found to be the strongest reacting, followed by those to CDw65 and CDw17 (Table I). Anti-CHO mAb stained granulocytes more brightly than mAb to adhesion molecules such as CD11b, CD18, CD35, and CD44 (Table I). Monocytes were negative for the anti-CD15 mAb and had

TABLE I

Binding of anti-CHO mAb to human leukocyte subsets

mAb	Ig Class	Ag	Form	MFI[a] Lymphocyte	MFI Monocyte	MFI Granulocyte	Source	Reference
PBS				1 ± 0	2 ± 0	2 ± 0		
MOPC 104 E	IgM	Negative control	Purified	1.0 ± 0	2 ± 0	2 ± 0	Sigma	
Dako-CD15	IgM	CD15	Supernatant	1 ± 0	3 ± 0	333 ± 43	DAKO	
My-1	IgM	CD15	Ascites	1 ± 0	5 ± 0	235 ± 21	C. I. Civin	(48)
AHN1.1	IgM	CD15	Ascites	1 ± 0	3 ± 1	260 ± 63	K. Skubitz	(19)
T5A7	IgM	CDw17	Supernatant	17 ± 9	20 ± 3	165 ± 26	F. Symington	(3)
G035	IgM	CDw17	Ascites	18 ± 2	10 ± 3	89 ± 24	J. Thompson	(49)
MEM-74	IgM	CDw17	Ascites	11 ± 2	15 ± 5	133 ± 10	V. Horejsi	(50)
B1B6	IgG1	CD43	Purified	ND	ND	ND	B. Axelsson	(52)
MEM-59	IgG1	CD43	Ascites	281 ± 10	282 ± 20	370 ± 26	V. Horejsi	(50)
84-3C1	IgG	CD43	Supernatant	163 ± 18	140 ± 12	230 ± 10	R. Vilella	(25)
HE10	IgM	CDw65	Ascites	32 ± 8[b]	43 ± 12	201 ± 12	M. Dockhelar	(51)
CF4	IgM	CDw65	Ascites	1 ± 0	2 ± 1	37 ± 1	M. Dockhelar	(51)
VIM-2	IgM	CDw65	Ascites	1 ± 0	93 ± 30	232 ± 9	Behring Werke	(51)
CSLEX1	IgM	s-CD15	Purified	34 ± 7[b]	77 ± 12	255 ± 23	P. Terasaki	(2)
FH6	IgM	s-CD15	Supernatant	31 ± 7[b]	54 ± 9	354 ± 100	F. Symington	(37)

[a] MFI, mean fluorescence intensity given as relative channel numbers (mean ± SEM; $n = 3$).
[b] Data given for subpopulation of positive cells (5–30%).

lower staining than granulocytes with all other anti-CHO mAb tested (Table I). In contrast, monocytes were brightly stained with mAb to CD43, CD44 and with the anti-CD45 mAb MEM-28 (Table II).

Cross-linking of carbohydrate Ag induces a cytoplasmic calcium flux independently of IgG FcR. Following the addition of GAM-M to cells labeled with ascites fluid of My1 (anti-CD15), there was a rapid and transient increase in cytoplasmic calcium concentration in granulocytes, but not in monocytes (Fig. 1; Table III). Monocytes did, however, respond with a calcium flux when GAM-HL was added to the same sample (Fig. 1). The monocyte response was abolished when IgG-depleted ascites fluid was used, whereas the granulocyte response was unchanged (Fig. 1). This suggests that the response of monocytes to GAM-HL was due to cross-linking of contaminating IgG antibodies, whereas the response of granulocytes to GAM-M was due to specific cross-linking of IgM mAb. As similar results were obtained with other mAb including AHN 1.1 (anti-CD15) and HE10 (anti-CDw65) (data not shown), and as GAM-M failed to induce calcium responses in cells labeled with several different IgG mAb that caused calcium fluxes when cross-linked by GAM-HL (data not shown), GAM-M was used throughout the study to avoid nonspecific IgG-FcR interactions.

When cells were labeled with a nonspecific IgM control antibody (MOPC-104E), no response was seen after the addition of any of the secondary antibodies (Fig. 1), whereas both monocytes and granulocytes gave positive control responses to 4 μM FMLP (Fig. 2). Treatment with FMLP yielded a 4.7 ± 0.3- and 5.0 ± 0.5-fold increase in mean Fluo-3 fluorescence of monocytes and granulocytes, respectively (mean ± SEM; $n = 5$). This increase was significantly greater than the response seen after cross-linking of CD15 ($p < 0.01$).

Cytoplasmic calcium fluxes in granulocytes were also seen after specific cross-linking of IgM mAb to s-CD15, CDw17, and CDw65 with GAM-M (Fig. 3, Table III). Monocytes responded only to cross-linking of one s-CD15 mAb, CSLEX-1 (Table III). This difference between the functional effects of two mAb to the same Ag could be due to low expression of the difucosyl-s-CD15 epitope preferred

TABLE II

Binding of mAb to glycoprotein Ag expressed by human leukocytes

mAb	Ig Class	Ag	Form	MFI[a] Lymphocyte	MFI Monocyte	MFI Granulocyte	Source	Reference
PBS				1 ± 0	2 ± 0	2 ± 0		
TEPC 183	IgM	Negative control	Ascites	1 ± 0	4 ± 0	3 ± 0	Sigma	
MOPC 104 E	IgM	Negative control	Purified	1 ± 0	2 ± 0	2 ± 0	Sigma	
MEM-25	IgG1	CD11a	Ascites	93 ± 14	137 ± 7	49 ± 6	V. Horejsi	(53)
Bear1	IgG1	CD11b	Ascites	21 ± 1[b]	136 ± 20	115 ± 8	Immunotech	
Mol	IgM	CD11b	Supernatant	12 ± 2[b]	90 ± 7	70 ± 2	Coulter	(54)
MEM48	IgG1	CD18	Ascites	33 ± 4	80 ± 5	57 ± 6	V. Horejsi	(53)
68-5A5	IgG	CD18	Supernatant	47 ± 4	118 ± 5	75 ± 6	R. Vilella	
J3D3	IgG1	CD35	Ascites	59 ± 5[b]	40 ± 8	36 ± 5	Immunotech	(55)
MEM-85	IgG1	CD44	Ascites	250 ± 50	490 ± 20	157 ± 9	V. Horejsi	(56)
BRIC-222	IgG1	CD44	Supernatant	287 ± 60	440 ± 20	207 ± 8	D. J. Anstee	
33-3B3	IgG	CD44	Supernatant	169 ± 8	314 ± 16	107 ± 10	R. Vilella	
MEM-28	IgG1	CD45	Ascites	485 ± 20	485 ± 122	129 ± 7	V. Horejsi	(50)
80H3	IgG1	CD67	Purified	29 ± 4[b]	17 ± 5	42 ± 3	Immunotech	

[a] MFI, mean fluorescence intensity given as relative channel numbers (mean ± SEM; $n = 3$).
[b] Data given for subpopulation of positive cells (5–30%).

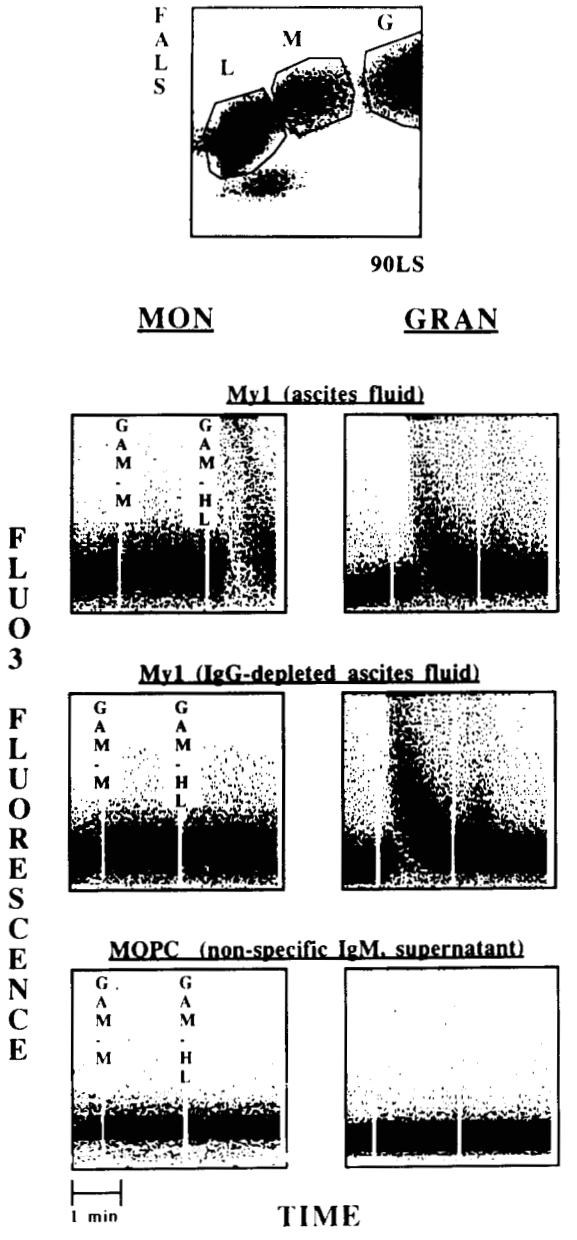

Figure 1. Flow-cytometric determination of fluctuations of leukocyte cytoplasmic calcium concentration after cross-linking of CD15 with mAb My1. *Upper diagram*: Leukocyte subsets identified by light scatter (L, lymphocytes; M, monocytes; G, granulocytes; 90LS, 90° light scatter; FALS, 4° light scatter). *Lower diagrams*: Linear Fluo-3 fluorescence of monocytes (MON) and granulocytes (GRAN) gated vs time. Fluo-3-loaded cells were labeled with indicated mAb preparation, washed, and resuspended in 200 μl PBS-FCS-Ca. To specifically cross-link the IgM mAb, 10 μl of GAM-M were added at indicated time point. To cross-link possible cell-bound IgG antibodies, 10 μl of GAM-HL were added at indicated time point. The diagrams are taken from an experiment representative of three performed.

by FH6 (37). When PBS was added instead of GAM-M, these rapid increases in cellular Fluo-3 fluorescence were never observed (data not shown).

Calcium flux induced by CHO cross-linking involves release of calcium ions from intracellular stores. To investigate the source of the calcium ions mobilized by CHO-cross-linking, granulocyte calcium fluxes were measured in the presence of the extracellular calcium chelator EGTA. These experiments showed that chelating of extracellular calcium ions did not abolish the calcium fluxes induced by cross-linking of granulocyte CHO-Ag (data not shown). This demonstrates that the observed responses at least partially are due to release of calcium ions from intracellular stores, and suggests that the phospholipase C/inositol trisphosphate pathway (38) could be involved in anti-CHO-induced activation.

Cross-linking of other Ag does not lead to cytoplasmic calcium fluxes. When nonspecific IgG FcR interactions were avoided by using $F(ab')_2$ fragments of IgG mAb, no significant calcium fluxes were seen after cross-linking of CD11a, CD18, CD35, CD43, or CD45 (data not shown). In the granulocyte population, there were no calcium fluxes induced by any mAb to CD11a, CD43, CD44, or CD45 even when whole IgG mAb were used (data not shown). The only mAb to other molecules that triggered FcR-independent calcium fluxes was the purified IgM anti-CD11b (Mol), which upon cross-linking with GAM-M induced a 3.1 ± 0.3- and 2.3 ± 0.3-fold increase in the Fluo-3 fluorescence of monocytes and granulocytes, respectively. In contrast, no response was triggered when cell-bound $F(ab')_2$ fragments of another anti-CD11b mAb (Bear1) were cross-linked with GAM-HL (data not shown).

Cross-linking of CHO Ag induces strong activation of the granulocyte respiratory burst. After addition of GAM-M to cells prelabeled with anti-CD15 (IgG-depleted ascites of My1) or anti-s-CD15 (FH6 supernatant), there was an increase in granulocyte Rhodamine 123 fluorescence as compared to granulocytes that had been incubated with PBS only (Fig. 4.; Table III). No response was seen when GAM-M was added to unlabeled cells or to cells that had been incubated with nonspecific IgM antibody (TEPC-183) (Fig. 4). Incubation of My1- or FH6-labeled cells in the absence of GAM-M did not result in increased Rhodamine 123 fluorescence above the PBS or irrelevant IgM controls (Fig. 4), demonstrating that cross-linking of the cell-bound IgM mAb was necessary for activation. Addition of FMLP to unlabeled or mAb-labeled cells also induced an increase in granulocyte Rhodamine 123 fluorescence (mean relative granulocyte Rhodamine 123 fluorescence, 5.5 ± 0.5; mean ± SEM, $n = 5$). This increase was, however, significantly smaller than the response to cross-linking of CD15 and s-CD15 with GAM-M in the same experiments ($p < 0.01$). No significant increase in the FMLP-induced respiratory burst was seen in cells prelabeled with mAb, suggesting that cells were not primed by potential contaminants in the antibody preparation (Fig. 4). No difference in the results was seen when IgG-containing ascites fluid of anti-CD15 mAb My1 was used (data not shown).

Similar results for granulocytes were obtained with other anti-CD15 and anti-s-CD15 mAb as well as with mAb to CDw17 and CDw65 (Table III) (data not shown). Monocytes responded with an increase in Rhodamine 123 fluorescence when incubated with CSLEX-1 (anti-sCD15) or VIM2 (anti-CDw65) followed by GAM-M but not with other mAb to these Ag (Table III).

Cross-linking of other Ag does not lead to activation of the respiratory burst. When FcR-dependent antibody binding was avoided by using $F(ab')_2$ fragments of IgG mAb, no significant respiratory bursts were seen after cross-linking of CD11a, CD18, CD35, CD43, or CD45 (data not shown). In the granulocyte population, there was no oxidative burst induced by cross-linking of any mAb to CD11a, CD35, CD43, CD44, or CD45, even when whole IgG mAb were used (data not shown). The only mAb to other molecules that triggered FcR-independent respiratory burst was the purified IgM anti-CD11b (Mol).

TABLE III

Monocyte and granulocyte activation after cross-linking of carbohydrate Ag (CD15, s-CD15, CDw17, CD43, CDw65)

Ag	Antibody	Calcium Flux		Oxidative Burst		Degranulation	
		Fluo-3 relative to control[a]		Rhodamine 123 relative to control[b]		CD11b-FITC relative to control[c]	CD67-FITC relative to control[d]
		Monocyte	Granulocyte	Monocyte	Granulocyte	Granulocyte	Granulocyte
CD15	My1	1.3 ± 0.3	2.8 ± 0.1[e]	1.3 ± 0.2	41.2 ± 6.0	2.2 ± 0.2	2.1 ± 0.1
	AHN 1.1	1.6 ± 0.2	3.3 ± 0.3	1.6 ± 0.3	24.4 ± 3.3	2.0 ± 0.1	1.7 ± 0.2
	DAKO15	1.1 ± 0.0	2.7 ± 0.3	1.1 ± 0.1	16.2 ± 3.2	2.0 ± 0.2	1.8 ± 0.1
s-CD15	FH6	1.4 ± 0.1	3.2 ± 0.3	1.1 ± 0.1	60.4 ± 7.2	2.1 ± 0.1	1.9 ± 0.1
	CSLEX1	3.2 ± 0.4	4.2 ± 0.5	2.0 ± 0.6	45.4 ± 6.0	2.1 ± 0.2	2.1 ± 0.1
CDw17	T5A7	1.3 ± 0.1	2.5 ± 0.3	1.1 ± 0.1	3.6 ± 0.5	1.4 ± 0.1	1.4 ± 0.1
	G035	1.3 ± 0.1	3.5 ± 0.4	1.5 ± 0.1	8.0 ± 3.0	1.8 ± 0.1	1.5 ± 0.1
	MEM74	1.2 ± 0.1	2.6 ± 0.5	1.2 ± 0.1	39.3 ± 5.3	1.9 ± 0.1	2.0 ± 0.1
CD43	MEM-59 F(ab)$_2$	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
	B1B6 F(ab)$_2$	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
CDw65	VIM2	2.8 ± 0.1	3.1 ± 0.3	2.6 ± 0.3	15.3 ± 4.0	1.8 ± 0.1	1.5 ± 0.2
	HE-10	1.6 ± 0.1	3.1±0.3	1.7±0.2	16.2±3.0	1.0±0.1	1.4±0.1
	CF4	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.2	1.8 ± 0.3	1.0 ± 0.1	1.1 ± 0.1

[a] The maximal Fluo-3 fluorescence after Ag cross-linking was divided by the mean fluorescence intensity before addition of F(ab')$_2$ fragments of goat antibodies. Results are given as mean ± SEM; $n > 4$.

[b] Rhodamine-123 fluorescence after Ag cross-linking was divided by the fluorescence intensity of unlabeled cells incubated with F(ab')$_2$ fragments of goat antibodies. Results are given as mean ± SEM; $n = 5$.

[c] Bear1 (anti-CD11b) FITC fluorescence after Ag cross-linking was divided by the fluorescence intensity of unlabeled cells incubated with F(ab')$_2$ fragments of goat antibodies. Results are given as mean ± SEM; $n = 5$.

[d] 80H3 (anti-CD67) FITC fluorescence after Ag cross-linking was divided by the fluorescence intensity of unlabeled cells incubated with F(ab')$_2$ fragments of goat antibodies. Results are given as mean ± SEM; $n = 5$.

[e] Underlined values, $p < 0.01$.

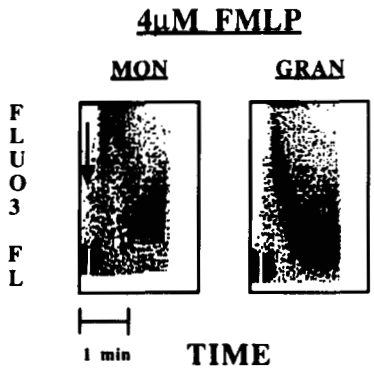

Figure 2. Flow-cytometric determination of FMLP-induced fluctuations of leukocyte cytoplasmic calcium concentration. Monocytes (MON) and granulocytes (GRAN) were recognized by light scatter and their Fluo-3 fluorescence (linear) was gated vs time. FMLP (4 μM) was added at the indicated time point. The diagrams are taken from an experiment representative of five performed.

Upon cross-linking of cell-bound Mo1 with GAM-M, there was a 2.5 ± 0.3- and 3.4 ± 0.5-fold increase in the Rhodamine 123 fluorescence of monocytes and granulocytes, respectively (mean ± SEM, $n = 5$), whereas no response was seen in the absence of GAM-M (data not shown). When F(ab')$_2$ fragments of another anti-CD11b mAb (Bear1) were applied in combination with GAM-HL, there was no increase in granulocyte or monocyte Rhodamine 123 fluorescence (data not shown), suggesting that extensive cross-linking is necessary for activation through CD11b on unprimed cells.

Staining and activation with anti-CDw17 mAb is decreased in FMLP-primed granulocytes. Stimulation of granulocytes with FMLP leads to a decrease in the expression of CDw17 and an increase the expression of CD11b (39, 40). To investigate whether the oxidative responses induced by mAb to these two Ag paralleled the cell surface Ag levels, we examined the effects of 2×10^{-7} M FMLP on staining and oxidative burst induced by MEM74 (anti-CDw17) and Mo1 (anti-CD11b). Priming with FMLP

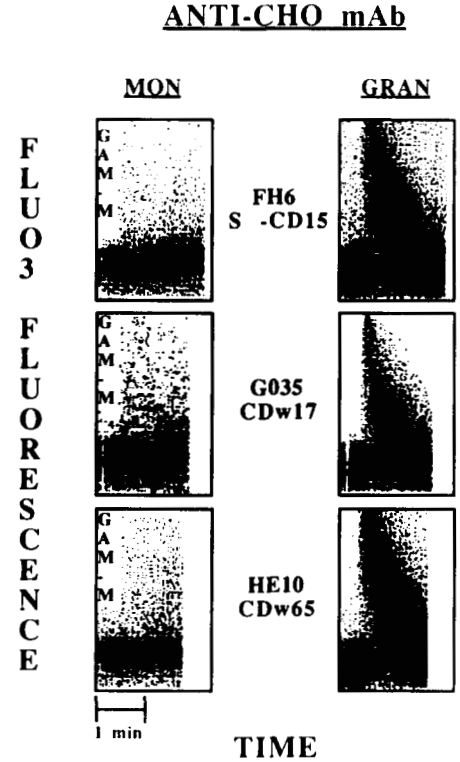

Figure 3. Flow-cytometric measurement of changes in monocyte (MON) and granulocyte (GRAN) calcium concentration after cross-linking of the carbohydrate Ag s-CD15, CDw17, and CDw65. The cells were prelabeled with the indicated mAb. Fluo-3 fluorescence (linear) was gated vs time for cell subsets recognized by light scatter. To specifically cross-link the IgM mAb, 10 μl of GAM-M were added at indicated time points. The diagrams are taken from an experiment representative of five performed.

led to a 50 ± 9% decrease in staining with anti-CDw17 mAb MEM-74 and a corresponding decrease (53 ± 8%) in the oxidative burst after cross-linking of cell-bound anti-CDw17 with GAM-M (mean ± SEM; $n = 3$) (Fig. 5). Typi-

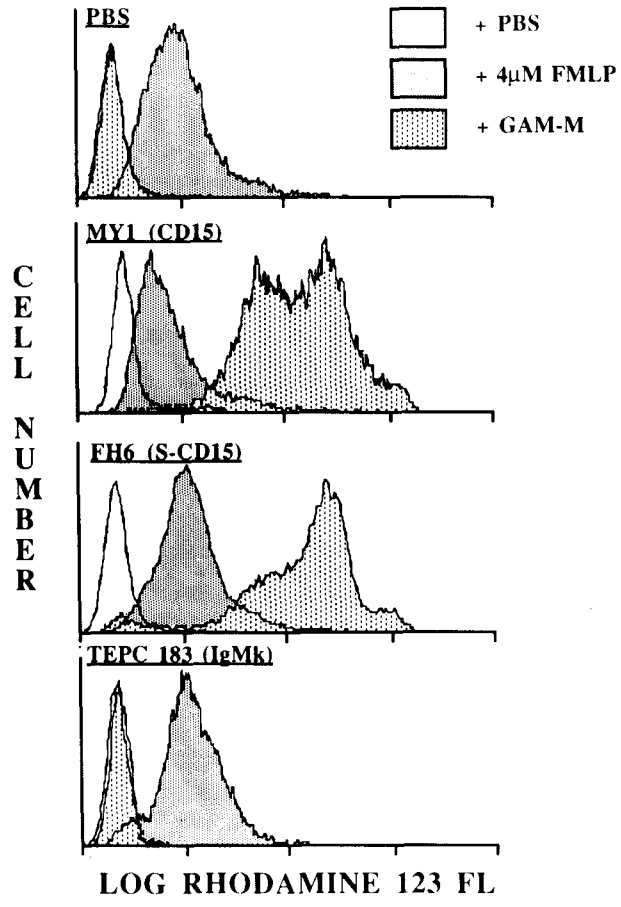

Figure 4. Flow-cytometric measurements of granulocyte oxidative burst after activation with anti-CHO mAb and FMLP. Unlabeled cells and cells labeled with the mAb indicated inside each panel were incubated 20 min at 37°C with PBS-FCS-Ca containing 10 μg/ml Dihydrorhodamine 123, no stimulus, GAM-M, or 4 μM FMLP. Granulocytes were recognized by light scatter measurements and their Rhodamine 123 fluorescence gated to one-parameter histograms. Histograms are taken from an experiment representative of five performed.

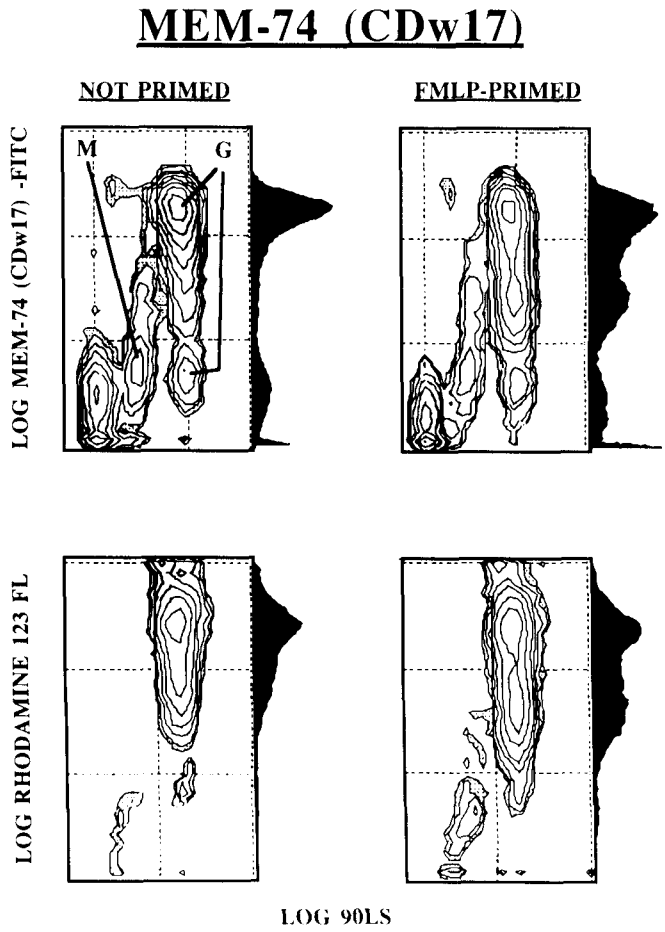

Figure 5. Flow-cytometric measurements of monocyte (M) and granulocyte (G) cell surface staining (*top*) and activation (*bottom*) with anti-CDw17. Cells were either primed (*left*) or not primed (*right*) with 2×10^{-7} M FMLP and labeled with anti-CDw17 (MEM-74). The cells were then either stained on ice with FITC-conjugated GAM-M (*top*) to measure cell surface CDw17 (*top*) or incubated at 37°C for 20 min with unconjugated GAM-M and 10 μg/ml Dihydrorhodamine 123 in PBS-FCS-Ca to assess oxidative burst (*bottom*). Fluorescence and 90° light scatter (90LS) (both log mode) were then measured for unprimed and primed cells. The *black histogram on the right border of each diagram* represents fluorescence intensity vs number of cells. The diagrams are taken from an experiment representative of three performed.

cally, granulocytes were heterogeneously affected by FMLP with regard to staining and oxidative burst with anti-CDw17 mAb (Fig. 5). In contrast to reduced staining and burst seen with anti-CDw17-primed granulocytes, priming with FMLP increased the staining and oxidative burst seen with the anti-CD11b mAb Mol. Priming with FMLP led to a 205 ± 40% increase in Mol fluorescence intensity as compared to nonprimed cells (mean ± SEM; $n = 3$) (Fig. 6). In addition, there was a 650 ± 80% increase in the granulocyte oxidative burst induced by the addition of GAM-M to Mol-labeled cells (mean ± SEM; $n = 3$) (Fig. 6). The background levels of Rhodamine 123 fluorescence were not altered by priming with FMLP, and in the absence of GAM-M, there was no increase in Rhodamine 123 fluorescence in any sample (data not shown). Therefore, oxidative responses induced by either anti-CDw17 or anti-CD11b required cross-linking of cell-bound mAb. In addition, these data demonstrate that the strength of the oxidative burst induced by cross-linking of CDw17 or CD11b parallels changes in cell surface expression of these Ag.

Respiratory burst induced by CHO cross-linking is associated with low up-regulation of CD11b and CD67. When changes in granulocyte CD11b expression and oxidative bursts were measured in the same experiments, cross-linking CD15, s-CD15, CDw17, and CDw65 led to a lesser increase in CD11b expression and a stronger oxidative burst than was observed after stimulation with 4 μM FMLP ($p < 0.01$) (Figs. 4 and 7). Stimulation with 4 μM FMLP led to a 3.1 ± 0.1-fold increase of granulocyte CD11b expression, whereas cross-linking of CHO Ag induced a doubling of granulocyte CD11b expression (Fig. 7, Table III). The difference between the responses induced by FMLP and anti-CHO-mAb was even larger when increases in granulocyte CD67 were measured. In FMLP-treated granulocytes, the mean CD67 fluorescence intensity was 5.5 ± 0.6 times higher than in cells incubated with PBS. In contrast, CD67 fluorescence typically only doubled after cross-linking of CHO Ag (Table III) ($p < 0.01$). Preincubation with mAb did not increase the FMLP-induced up-regulation of either CD11b or CD67 (Fig. 7) (data not shown).

DISCUSSION

We have demonstrated that cross-linking of CHO Ag on human granulocytes and monocytes induces cytoplasmic calcium fluxes, activation of the respiratory burst, and increased cell surface expression of granule-associated proteins. Several lines of evidence suggest that the observed responses reflect functional characteristics

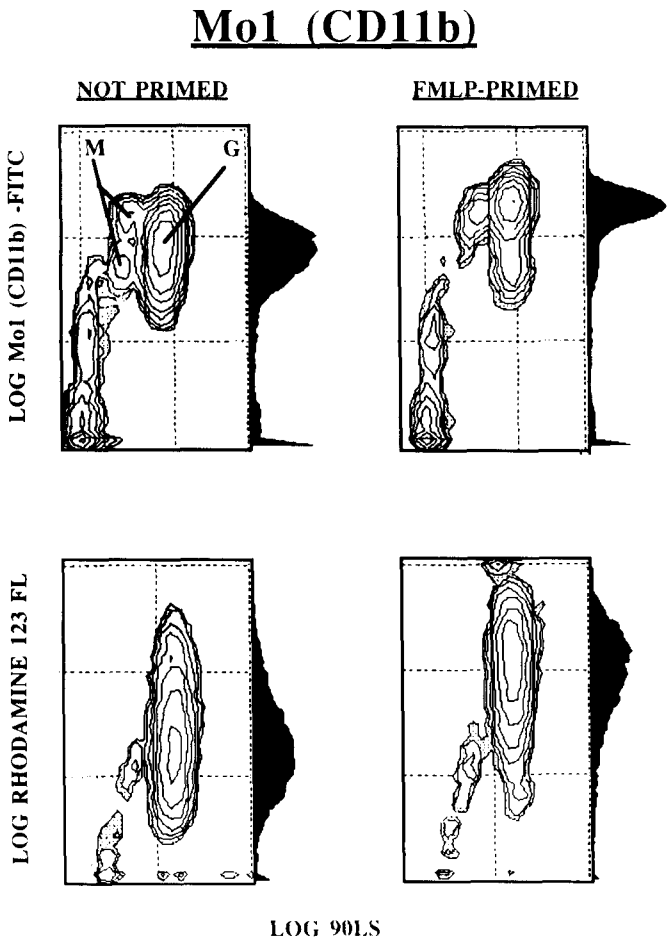

Figure 6. Flow-cytometric measurements of monocyte (M) and granulocyte (G) staining (*top*) and activation (*bottom*) with anti-CD11b mAb Mo1. Cells were either (*right*) or not (*left*) primed with 2×10^{-7} M FMLP and labeled with anti-CD11b. The cells were then either stained on ice with FITC-conjugated GAM-M (*top*) to measure cell surface CD11b (*top*) or incubated at 37°C for 20 min with unconjugated GAM-M and 10 μg/ml Dihydrorhodamine 123 in PBS-FCS-Ca to assess oxidative burst (*bottom*). Fluorescence and 90° light scatter (90LS) (both log mode) were then measured for unprimed and primed cells. The *black histogram on the right border of each diagram* represents fluorescence intensity vs number of cells. The diagrams are from the same experiment as those in Figure 5 and are representative of three performed.

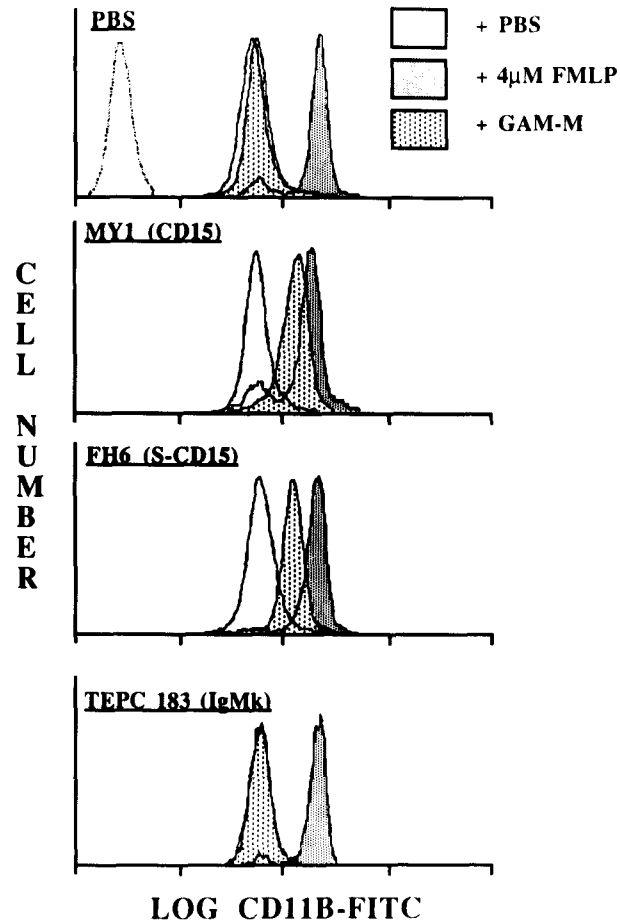

Figure 7. Flow-cytometric measurements of cellular CD11b expression after activation with anti-CHO mAb and FMLP. Unlabeled cells and cells labeled with indicated mAb (Ag in *parentheses*) were incubated 20 min at 37°C with PBS-FCS-Ca alone or with PBS-FCS-Ca containing GAM-M or 4 μM FMLP. The cells were then labeled with FITC-anti-CD11b (Bear1). Granulocytes were recognized by light scatter measurements and their CD11b-FITC fluorescence gated to one-parameter histograms. The *dotted curve at the extreme left in the upper panel* shows the binding of a FITC conjugated nonspecific control mAb. Histograms are taken from the same experiment as those shown in Figure 4, and are representative of five experiments.

that are specific for CD15, s-CD15, CDw17, and CDw65. 1) Cell activation induced by CHO cross-linking was not due to FcR interactions, inasmuch as IgM mAb and IgM-specific secondary antibodies were used. 2) Specific binding of the mAb to the cells was necessary for activation. 3) No activation was seen unless the cell-bound mAb was cross-linked by a secondary antibody. 4) Activation could be reproduced with more than one mAb to each of these CHO Ag, but not with mAb specific for a number of other Ag including the high density CHO-epitope of CD43 (leukosialin). 5) FMLP-induced decrease in staining with anti-CDw17 was followed by a similar decrease in the ability of anti-CDw17 mAb to induce cell activation. 6) Preincubation with mAb did not alter the oxidative burst or increase in cell surface expression of CD11b induced by 4 μM FMLP, demonstrating that the cells were not nonspecifically primed by potential contaminants in the mAb preparations. The results, therefore, suggest that CD15, s-CD15, CDw17, and CDw65 can act as receptor molecules capable of mediating calcium fluxes, degranulation, and strong activation of the oxidative burst in granulocytes.

Our results support and extend earlier reports suggesting involvement of the CHO Ag in initiation of granulocyte activation. mAb to the CD15 molecule have been shown to inhibit the oxidative burst induced by zymosan particles (17, 19, 22) and to inhibit phagocytosis of IgG- and C-opsonized particles and bacteria (18, 19). Similarly, mAb to CDw17 and CDw65 have been found to inhibit the zymosan-induced respiratory burst (22), and anti-CDw17-coated bacteria are specifically phagocytosed and halogenated by human neutrophils (41). In addition to these effects, mAb to CD15 have also been found to cause activation of granulocytes, inducing responses such as a cytoplasmic calcium flux (20), degranulation (21), cell movement (21), and increased adhesion to endothelium (24). Furthermore, Sullivan et al. (23) reported that $F(ab')_2$ fragments of the IgG3 anti-CD15 mAb PMN7C3 (IgG3) could trigger an oxidative burst in GM-CSF-activated granulocytes. Attempts to trigger the oxidative burst by CD15 mAb in unprimed cells have, however, failed (21). The present study demonstrates an oxidative burst in unstimulated cells mediated by CD15, as well as s-CD15, CDw17, and CDw65, and supports the view that these Ag are involved in initiating cell activation. The reason for the discrepancy in the results from this and earlier studies could be that the combination of IgM mAb

and IgM-specific cross-linking antibodies used in this study may have caused more extensive aggregation of CHO molecules than has been obtained in earlier studies. This hypothesis is supported by the results showing that mAb alone was without effects, and that the IgM mAb Mol to CD11b caused calcium fluxes and oxidative bursts, whereas $F(ab')_2$ fragments of an IgG mAb to the same molecule failed to do so.

The mechanism by which anti-CHO mAb induces cell activation remains to be determined. The rapid release of calcium ions after cross-linking suggests that anti-CHO mAb may activate phospholipase C as does FMLP (38). The increase in cytoplasmic calcium concentration is, however, not likely to be responsible for all of the respiratory burst activity, inasmuch as FMLP induced calcium fluxes of higher amplitude than anti-CHO mAb, but induced less oxidative burst activity. In addition, FMLP caused more extensive degranulation than anti-CHO mAb. These differences in the response patterns of FMLP and anti-CHO mAb indicate that these stimuli may activate qualitatively different signaling pathways. This subject is presently being investigated.

The mAb to CD15 bind to carbohydrates present on glycolipids and glycoproteins including CD11b (42, 43) and CD35 (43, 44). Similarly, mAb to s-CD15 bind to epitopes present glycolipids and on the cell adhesion molecule LECAM-1 (4, 45). Inasmuch as the CD11b molecule was found to be capable of mediating cell activation when cross-linked by Mol and GAM-M, some of the effects seen with anti-anti-CD15 mAb might be ascribed to cross-linking of this glycoprotein. Several lines of evidence suggest, however, involvement of other structures than C receptors in mediating activation by anti-CHO mAb. 1) No intracellular responses were seen after cross-linking of CD18 (which is associated with CD11b) or CD35. 2) Cross-linking of CHO Ag led to significantly stronger activation of the respiratory burst than cross-linking of CD11b. 3) Priming of cells with FMLP, which down-modulates granulocyte expression of CDw17 (39), led to a specific decrease in the oxidative burst induced by anti-CDw17, thereby strengthening the view that the glycolipid, lactosylceramide, is involved in activation by anti-CDw17. The intracellular events observed after cross-linking of CD15, s-CD15, CDw17, and CDw65 may therefore reflect engagement of CD11b as well as glycolipids and glycoproteins with as yet undescribed functions.

Whereas activating effects of mAb may indicate a link between an Ag and a specific function, the recognition of physiologic ligands capable of triggering similar cellular responses is necessary to classify the molecule as a receptor. It is therefore interesting that the interaction of granulocytes with a physiologic ligand for CHO Ag, the ELAM-1 molecule, leads to enhanced adhesive properties of their membrane integrins (46) similar to what has been described for anti-CD15 mAb (24). As CHO-mediated adhesion to ELAM-1 on activated endothelium most likely leads to multivalent engagement of s-CD15 or CDw65 on the granulocyte surface, membrane alterations like those occurring during antibody cross-linking may be induced. Aggregates of CHO Ag may also be formed when bacteria with binding sites for lactosylceramide (CDw17) (47) interact with granulocyte membranes or when GMP-140 (CD62) on activated platelets bind to granulocyte CD15 or s-CD15. Therefore, the nature of the ligands for the CHO molecules investigated in this study favors the hypothesis that cross-linking is a physiologic phenomenon that triggers cell activation.

In conclusion, the present study demonstrates that transmembrane signaling in human granulocytes and monocytes can be triggered by cross-linking CHO Ag. The results strengthen the view that these molecules and these CHO and the molecules that bear them are important for phagocyte activation. Additional studies using the newly characterized ligands for CD15, s-CD15, CDw17, and CDw65 may reveal whether or not the stimulatory effects of mAb reflect the biologic roles of these Ag.

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