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# Serum Factors and the Cellular Redox Status Regulate Cellular Responsiveness to MHC Class II-Triggered Homotypic B Cell Adhesion

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**Cross-linking the major histocompatibility complex (MHC) class II (MHC-II) by their cognate ligands including mAb induces homotypic cell adhesion. It has been shown that merely surface MHC-II expression is insufficient to induce the response. We found that MHC-II-triggered cell adhesion in human B cell lines was absent when serum was removed from medium. Even in the presence of serum, the response was prevented when cells were treated with the glutathione synthesis inhibitor, buthionine sulfoximine, an irreversible catalase inhibitor, aminotriazole, and $H_2O_2$. Flow cytometrical analysis showed that these changes in cellular responsiveness were unlikely to be merely the result of altered surface Ag expression. In addition, the response was independent of the two major lymphocyte adhesion receptors, LFA-1 and intercellular adhesion molecule-1 (ICAM-1). These findings suggest that serum- and redox-sensitive intracellular events regulate cellular responsiveness to MHC-II-triggered LFA-1/ICAM-1-independent cell adhesion independently of differentiation.** 

Engagement of the major histocompatibility complex (MHC) class I and class II (MHC-I and MHC-II) by the cognate ligands such as T cell receptor/CD4, bacterial superantigens, and monoclonal antibodies (mAbs) can induce homotypic cell adhesion in B and T cells, and monocytes (1-9). The response is mediated by LFA-1 (CD11a/CD18)/intercellular adhesion molecule-1 (ICAM-1,CD54) interactions as well as by other unidentified adhesion molecules (1-9). The response has been shown to be inhibited by metabolic depletion of cellular ATP as well as by inhibitors of cytoskelton but not inhibited by DNA, RNA, and protein synthesis, indicating the requirement of cellular energy and an appropriate cytoskelton structure but not *de novo* protein synthesis (2,5). Experiments using a range of protein kinase inhibitors show that the LFA-1-dependent pathway is protein kinase C (PKC)-dependent, while the LFA-1-independent pathway is PKC-independent and protein tyrosine kinase (PTK)-dependent (2-8). Taken together, these observations suggest that MHC-II ligation generates intracellular signals, which lead to the activation of LFA-1 and/or other adhesion molecules through protein kinase activation and changes in cytoskelton structure.

Binding between MHC-II molecules and their ligands is essential to induce cell adhesion. Therefore, negative responses correlate with the absence of expression of that molecule. However, it has been shown that MHC-II-triggered cell adhesion lacks a strict correlation between surface MHC-II expression and the intensity of the response (2-4). In particular, some cell types exhibit no cell adhesion in response to MHC-II stimulation in spite of the higher levels of surface MHC-II expression. Furthermore, the difference in the response can not be explained solely by differences in epitope density or isotype differences (2-4). These observations indicate that merely surface MHC-II expression is insufficient to induce cell adhesion response. The fact strongly suggests that as yet undefined another mechanisms are important in the generation of cell adhesion signal(s). Here we demonstrate evidence that serum- and redox-sensitive intracellular events regulate cellular responsiveness to MHC-II-triggered B cell adhesion independently of differentiation.

## MATERIALS AND METHODS

*Reagents and mAb.* $H_2O_2$ was obtained from Wako Pure Chemical (Osaka, Japan). L-buthionine-S,R-sulfoximine (BSO), luminol, horseradish peroxidase, and 3-amino-1,2,4-triazole (ATZ) were from Sigma (St. Louis, MO). The following three mAb to MHC-II were all obtained from Dako (Glostrup, Denmark). CR3/43: $\beta$ chain of all DP, DQ, DR; DK22: $\beta$ chain of all DR, DP, DQw1 but not DQw3; TAL.1B5:

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$\alpha$ chain of DR. The anti-LFA-1 mAb 25.3.1 and the anti-ICAM-1 mAb LB-2, both of which are suitable for adhesion blockade were from Immunotech (Marseilles, France) and Becton Dickinson (Mountain View, CA), respectively.

*Cells.* BJAB and Ramos are human B-cell lymphoma lines. These cells were cultured at 37°C in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Mitsubishi Kasei, Tokyo, Japan), 100 units/ml penicillin, and 100 $\mu$g/ml of streptomycin in a humidified atmosphere containing 5% $CO_2$/95% air.

*Determination of cell adhesion.* Cell adhesion was determined according to the method of Kansas and Tedder (2) with minor modifications. Briefly, cells were suspended with RPMI 1640 medium supplemented with or without 10% FCS and placed in a 96-well tissue culture plate. Cells were added with 1 $\mu$g/ml of each mAb and incubated at 37°C for the time indicated. Semi-quantitative scoring of adhesion was carried out as follows. 0, no adhesion or cell cluster formation (<90% of the cells were unaggregated); 1, >20% of the cells were aggregated, although the majority of the cells were unaggregated; 2, about 50% of the cells were aggregated; 3, nearly all cells were aggregated with only a few (<20%) unaggregated cells; 4, <90% of the cells were in large aggregates.

*Determination of cell viability.* Cell viability was determined by WST-1 assay, a modified MTT assay (10) as recommended by the supplier (Dojin Laboratories, Kumamoto, Japan). In the assay, a compound that releases a soluble formazan product was used to quantitate mitochondorial enzymes in living cells. Briefly, cells were cultured for 18 hrs in the presence or absence of the agents tested. Next, the mixture of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tertazolium monosodium salt) and 1-methoxy-5-methylphenazinium methylsulfate was added. After incubation for 2 hrs, absorbance at 410 nm was measured in a multiplate reader using absorbance at 690 nm as a reference.

*Measurement of intracellular ROS.* The effect of redox-modulating agents on intracellular ROS levels was determined by monitoring chemiluminescence, a strong indicator of ROS as previously described (11). Briefly, the assay mixture (0.25 ml) contained Hank's balanced salt solution (HBSS) without phenol red, 8 mM luminol and horseradish peroxidase (1U/ml), and $3 \times 10^5$ cells. Assay components were added to cuvettes, mixed and placed immediately in a luminometer (Bio-Lumat LB9507, Berthold, Wildbad, Germany). After 3 min preincubation, the reaction was started by the addition of 0.2 mM menadione. At 20 sec intervals up to 12 min the CL emission from the samples was recorded. The results were expressed as relative light units (RLU)/sec where 1 RLU represents 10 photon counts. When the effect of $H_2O_2$ and ATZ on spontaneous or menadione-evoked ROS generation (12), cells were preincubated for 30 min with the agents tested at the doses indicated.

*Flow cytometrical analysis of surface antigens.* The expression of surface molecules was assessed by flow cytometry. Briefly, $10^6$ cells were incubated at 4°C for 1 hr with the relevant mAb and then incubated at 4°C for 30 min with FITC-conjugated anti-mouse Ig. After extensively washing with PBS, the samples were analyzed in a cytometer (FACScan, Bectone Dickinson, Mountain View, CA).

## RESULTS

### *Serum-Dependence of MHC-II-Triggered Cell Adhesion*

The human B cell lymphoma line, BJAB ($2 \times 10^5$/ml) were seeded in the medium supplemented with 10% FCS and incubated for 18 hrs with mAb directed against MHC-II and cell adhesion was determined microscopically as described in Materials and Methods. Consistent with the previous reports using physiological ligands and other mAb (2-9), the addition of several mAbs resulted in cell adhesion. The mAb used in the present study commonly recognize DR locus of MHC-II, which is consisted of two distinct polypeptide chains, $\alpha$ and $\beta$. The two mAbs (CR3/43 and DK22) that recognize $\beta$ chain, were potent inducers of cell adhesion, while one mAb (TAL.1B5) recognizing only $\alpha$ chain was without effect. On the other hand, isotype-matched Igs were without effect. Basically the same results were obtained with Ramos B cells. The response could be observed as early as within 3 hrs following the addition of each mAb, reaching a maximum at 18 hrs, and observed during at least another 48 hrs.

Although anti-MHC-II reproducibly induced cell adhesion response, a considerable variation in the extent of the effect was observed between separate experiments even under the same experimental conditions. The observations prompted us to investigate the effect of cell growth conditions on the response. To determine the effect of serum starvation on anti-MHC-II-induced cell adhesion, cells were suspended in the medium supplemented with or without 10% FCS and cultured for 18 hrs with anti-MHC-II. When unstimulated, BJAB cells were growing without making cell clusters (Fig. 1, panel A). As described above, addition of the anti-MHC-II mAb CR3/43 to the cells resulted in cell adhesion (Fig. 1, panel B). By sharp contrast, when serum was removed from the medium, the addition of the mAb induced no cell adhesion (Fig. 1, panel E), although no significant changes in cell morphology (Fig. 1, panel D), cell viability, or surface MHC-II expression was observed (data not shown). Similarly, another anti-MHC-II mAb DK22 induced cell adhesion in the presence of serum (Fig. 1, panel C) but not in the absence of serum (Fig. 1, panel F). Similar results were obtained Ramos, too.

### *Inhibition of MHC-II-Triggered Cell Adhesion by Redox-Modulating Agents*

Serum contains various thiol-containing substances that act as antioxidants and protect cells from oxidative injury. Therefore, there was the possibility that the serum-dependence of MHC-II-triggered cell adhesion might result from the requirement of certain oxidants and/or an appropriate cellular redox status in the response. To assess the causal relationship between the cellular redox status and the adhesion capacity of the cells, we determined the effect of altered cellular redox status on the adhesion. We noticed that the induction of cell adhesion by anti-MHC-II was more evident in high density cells than in low density cells. Therefore, to obtain reliable results, experiments were performed with cells at a high density ($1 \times 10^6$/ml). We first tested the effect of BSO on anti-MHC-II-induced cell adhesion. The agent specifically inhibits $\gamma$-glutamylcys-

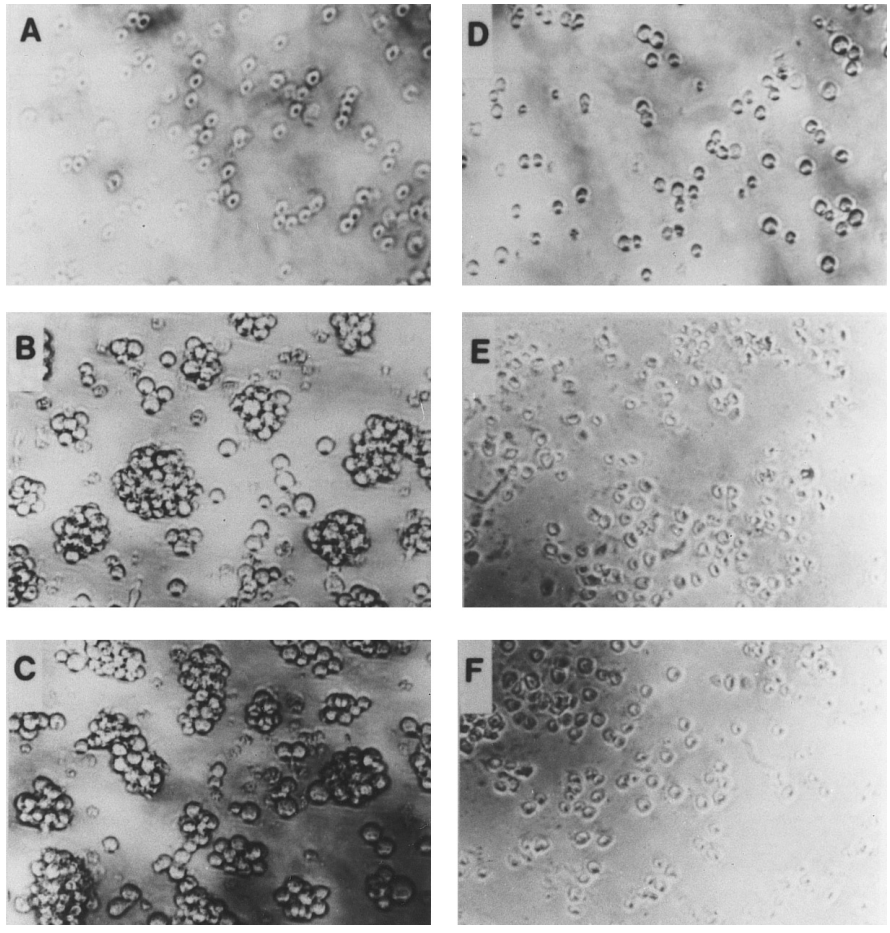



**FIG. 1.** Serum-dependent induction of B cell adhesion by anti-MHC-II. BJAB ($2 \times 10^5$/ml) were seeded in the medium supplemented with (panel A,B,C) or without 10% FCS (panel D,E,F) and incubated with medium (panel A,D) or 1 $\mu$g/ml of the anti-MHC-II mAb CR3/43 (panel B,E) and DK22 (panel C,F), and cell adhesion was determined microscopically as described in Materials and Methods. Photographs were taken at 18 hrs after the addition of each mAb.

teinyl synthetase, an essential enzyme in the synthesis of glutathione (GSH) (13,14). Because GSH is the major antioxidant in every cell types (reviewed in 13), the decrease in intracellular amounts of the substance was expected to cause the shift in the cellular redox status to oxidizing redox status. In fact, we have previously shown that treatment of these B cells with BSO augments $H_2O_2$-induced tyrosine phosphorylation by decreasing intracellular GSH levels (15). When the cells were pretreated with 100 $\mu$M BSO for 18 hrs, the BSO treatment inhibited anti-MHC-II-induced cell adhesion in both BJAB and Ramos. Both cell adhesion score and the size of aggregates were evidently reduced in the BSO-treated cells when compared with those observed in the control cells (Table 1). The BSO treatment showed a minimal effect on cell growth or surface MHC-II expression (data not shown).

Because catalase is a scavenger of $H_2O_2$, the inhibition of the antioxidant enzyme was also expected to cause the shift in the cellular redox status to oxidizing redox status. As shown in Fig. 2, catalase inhibition by an irreversible inhibitor ATZ (16) enhanced CL response, a strong indicator of ROS in a dose-dependent manner. The effect was more evident when intracellu-

**TABLE 1**

Effect of BSO on Anti-MHC-II-Induced Cell Adhesion

| Cells | mAb | BSO(−) | BSO(+) |
|---|---|---|---|
| BJAB | CR3/43 | +++ | + |
| | DK22 | +++ | + |
| Ramos | CR3/43 | ++ | − |
| | DK22 | ++ | − |

*Note.* Cells resuspended at a cell density of $1 \times 10^6$/ml in RPMI 1640 medium supplemented with 10% FCS were incubated for 18 hrs with 100 $\mu$M BSO and then treated with 1 $\mu$g/ml of anti-MHC-II mAbs, and cell adhesion was determined microscopically as described in Materials and Methods. Results are representative of three separate experiments.

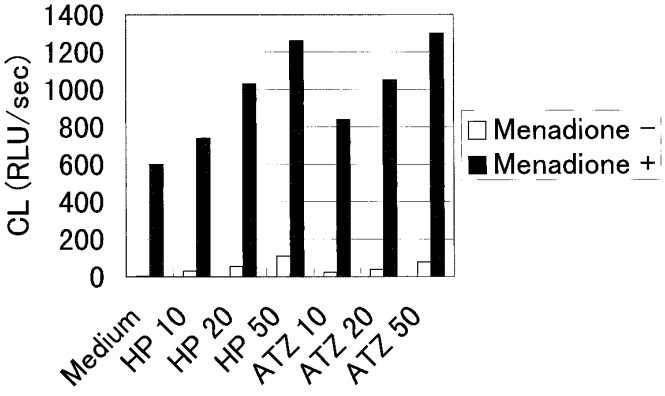



**FIG. 2.** Effect of ATZ and $H_2O_2$ on intracellular ROS levels. BJAB cells were preincubated for 30 min with ATZ (10, 20, and 50 mM) or $H_2O_2$ (HP, 10, 20, and 50 $\mu$M) and spontaneous (menadione-) or menadione-evoked CL response was measured for 12 min as described in Materials and Methods. Menadione-evoked CL response peaked at 10 min after the addition of menadione and the data represent the peak values of CL observed in each sample. The data are representative of three separate experiments with the same results.

lar ROS levels increased in response to menadione, which generates superoxide intracellularly and extracellularly (12). These results indicate that as one expected, catalase inhibition increases intracellular ROS.

Then, the effect of ATZ on anti-MHC-II-induced cell adhesion was examined. Since the agent showed a significant cytotoxicity upon a longer (18 hrs) exposure but not upon a shorter (3 hrs) exposure, we employed the latter non-cytotoxic conditions to avoid any secondary effect due to cytotoxicity. The agent inhibited the response in a dose-dependent manner (Figs. 3A and 3B). The response induced by DK22 was more resistant than that induced by CR3/43. Both responses were completely inhibited by 50 mM ATZ that was comparable to the dose required for catalase inhibition. Basically the same results were obtained with BJAB and Ramos.

To elucidate the involvement of the cellular redox status more directly, the effect of exogenous $H_2O_2$ on anti-MHC-II-induced cell adhesion was also examined. $H_2O_2$ is a membrane-permeable oxidant and therefore it readily enters into cells and increased intracellular ROS when added exogenously as shown in Fig. 2. Treatment with the oxidant inhibited the response in a dose-dependent manner (Fig. 3C), although it did not affect significantly cell growth (data not shown). Also in this case, the response induced by DK22 was more resistant than that induced by CR3/43. The results are similar to those obtained with ATZ, indicating that ATZ and the oxidant inhibit the response through a similar mechanism. Flow cytometrical analysis showed that even when anti-MHC-II-induced cell adhesion was completely prevented (see, Figs. 3A and 3B), a substantial amount of MHC-II was expressed on cell surfaces (Fig. 3D). Taken together, these results demonstrate that these redox-modulating agents prevent MHC-II-triggered cell adhesion without impairing surface MHC-II expression.

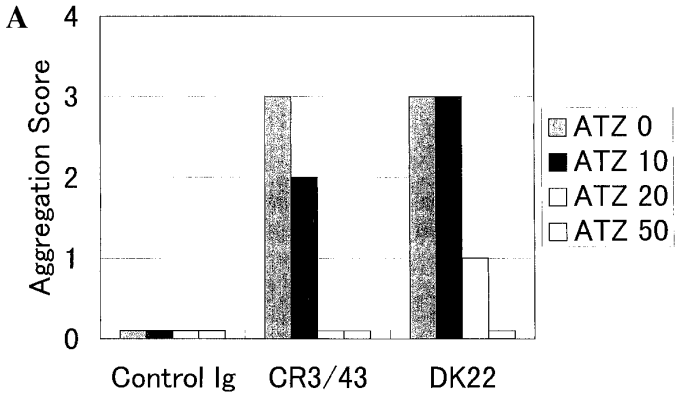
### *LFA-1 and ICAM-1-Independence of MHC-II-Triggered Cell Adhesion*

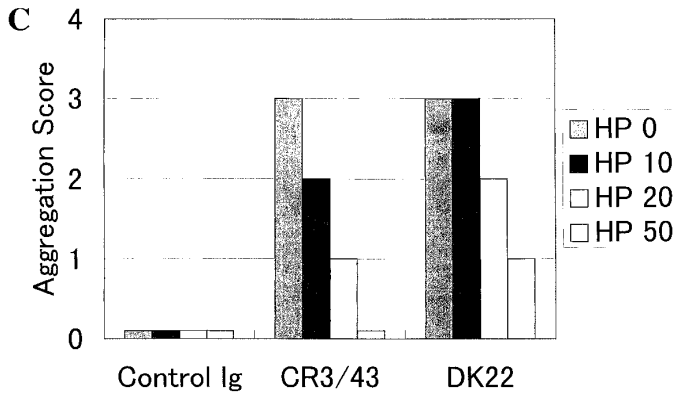
Because the interactions between LFA-1 and ICAM-1 play a major role in mediating homotypic and heterotypic cell adhesion in lymphocytes, the involvement of these adhesion receptors in anti-MHC-II-induced cell adhesion was investigated. Flow cytometrical analysis showed that in both BJAB and Ramos, a substantial amount of the LFA-1 ligand, ICAM-1 was expressed (Fig. 4B and 4D), while the expression of LFA-1 on their surfaces was below detectable levels (Figs. 4A and 4C). In both BJAB and Ramos cells, direct staining using FITC-conjugated anti-LFA-1 mAb also failed to detect significant binding, indicating that the expression of LFA-1 on their surfaces was if any, exclusively low. Thus, anti-MHC-II-induced adhesion of these B cells is unlikely to be LFA-1-dependent.

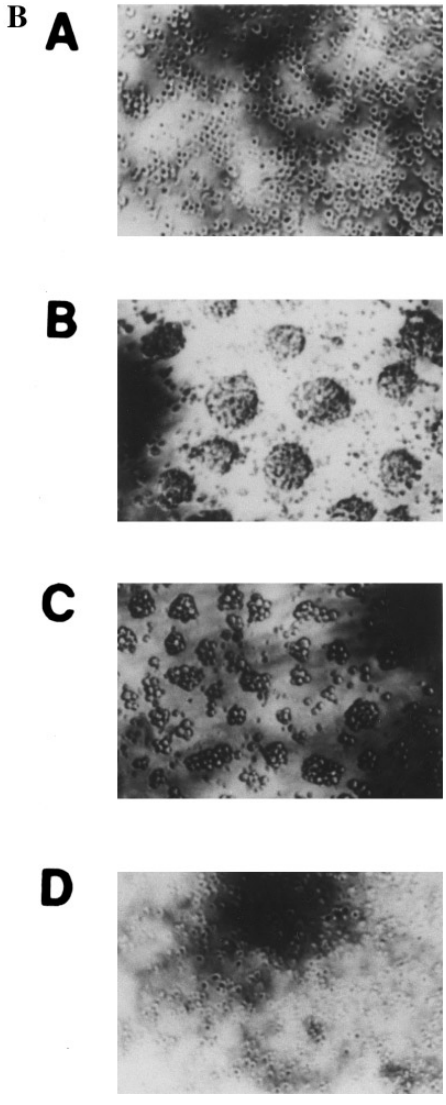
To ensure this conclusion, we carried out blocking experiments. Both of the anti-LFA-1 mAb 25.3.1 and the anti-ICAM-1 mAb LB-2 were shown to inhibit the LFA-1-ICAM-1 interactions in various cell types (17,18). In fact, we have recently observed that both mAb completely inhibit heterotypic cell adhesion between LFA-1-expressing EBV-infected BJAB subline, BJAB/B95-8 and ICAM-1-expressing human corneal epithelial cells (unpublished results). Cells were treated with 1 $\mu$g/ml of anti-MHC-II mAb in the presence or absence of 10 $\mu$g/ml of either blocking mAb or both. Neither mAb alone nor in combination effectively inhibited the cell adhesion (Table 2). In addition, EDTA that is shown to prevent cell adhesion mediated through integrins, also failed to inhibit the cell adhesion (Table 2). The results demonstrate that MHC-II-triggered cell adhesion is LFA-1/ICAM-1-independent.

## DISCUSSION

The present study demonstrates that serum starvation and altered cellular redox status prevent MHC-II-triggered B cell adhesion independently of surface MHC-II expression. Interestingly, it has been shown that MHC-II-triggered cell adhesion lacks a strict correlation between surface MHC-II expression and the intensity of the response. MHC-II on some cell types including pre-B cell lines fail to transduce cell adhesion response (2,3). Because pre-B cells specifically show such unresponsiveness in the differentiation stage of B cells, the failure of the response has been thought to result from a lack or dysfunction of certain cellular machinery essential for the response at particular differentiation stage. However, here we demonstrate that the failure of MHC-II-triggered cell adhesion can be



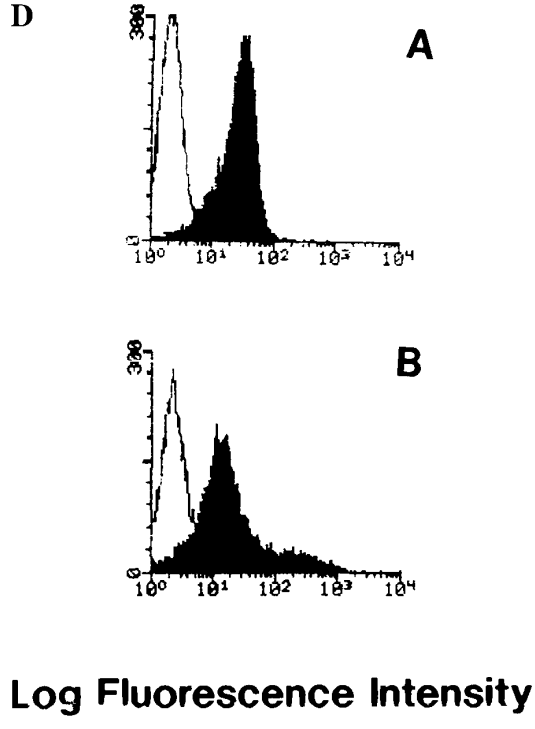




**FIG. 3.** A. Effect of ATZ on anti-MHC-II-induced cell adhesion. BJAB cells ($1 \times 10^6$/ml) suspended in the medium supplemented with 10% FCS were incubated with 1 $\mu$g/ml of the anti-MHC-II mAb CR3/43 or isotype-matched Ig (control Ig) in the absence or presence of 10, 20, and 50 mM ATZ and cell adhesion was determined microscopically. B. Effect of ATZ on cell morphology. BJAB cells ($1 \times 10^6$/ml) suspended in the medium supplemented with 10% FCS were incubated with medium (panel A) or 1 $\mu$g/ml of the anti-MHC-II mAb CR3/43 (panels B,C,D) in the presence of medium (panel B), 20 mM ATZ (panel C), and 50 mM ATZ (panel D). Photographs were taken at 18 hrs after the addition of each mAb. C. Effect of exogenous $H_2O_2$ on anti-MHC-II-induced cell adhesion. BJAB cells were incubated with 1 $\mu$g/ml of the anti-MHC-II mAb CR3/43 or isotype-matched Ig (control Ig) in the absence or presence of 10, 20, and 50 $\mu$M $H_2O_2$ and cell adhesion was determined as described above. D. Flow cytometrical analysis of surface MHC-II expression. BJAB cells were incubated for 3 hrs in the absence (A) or presence of 50 mM ATZ (B) and the treated cells were stained with the anti-MHC-II mAb CR3/43 (shaded histograms) or the isotype-matched Ig (open histograms), followed by FITC-conjugated secondary antibody.

induced in a single cell type, indicating that it is independent of differentiation. Thus, besides surface MHC-II expression, another cellular event(s) sensitive to growth conditions may play an essential role in the cell adhesion response. Serum delivers cell proliferating or survival signals. However, the failure of MHC-II-triggered cell adhesion could be observed without altered cell proliferation. Furthermore, Consistent with several previous reports with other mAbs (3,19,20), an anti-MHC-II mAb (DK22) induced growth inhibition in our system. Therefore, the serum-dependence of MHC-II-triggered cell adhesion seems to be separated from these growth-related signals.

Even in the presence of serum, both increasing cellular ROS levels and decreasing antioxidant levels caused the failure of MHC-II-triggered cell adhesion without affecting surface MHC-II expression. These observations suggest a critical role of the cellular redox status in generating intracellular signals for the response, although the point of the redox regulation is at the present unclear. The cellular redox status is shown to play an important role in gene expression by regulating DNA binding and/or activation of several transcription factors such as NF-$\kappa$B (21) and AP-1 (22). However, the redox modulation of MHC-II-triggered cell adhesion may be separated from such regulation of gene expression because the response does not require any gene expression.

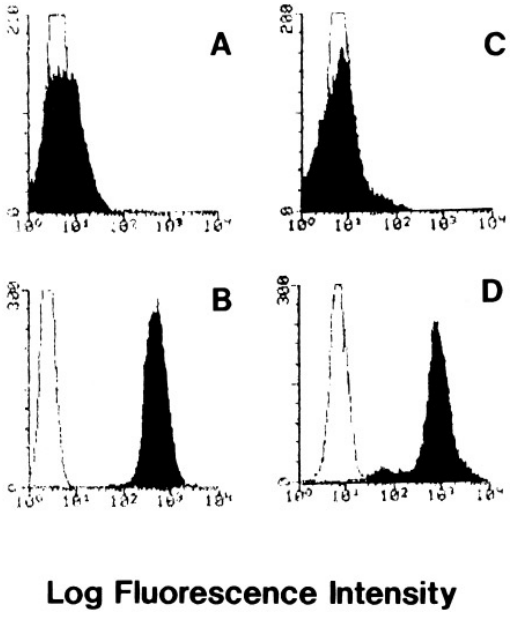



**FIG. 4.** Flow cytometrical analyses of the expression of LFA-1 and ICAM-1 on cell surfaces. The expression of LFA-1 (A,C) and ICAM-1 (B,D) on the surfaces of BJAB (A,B) and Ramos cells (C,D) were determined by flow cytometry using the anti-LFA-1 mAb 25.3.1 and the anti-ICAM-1 LB-2 as described in Materials and Methods. shaded histograms: staining with the anti-LFA-1 or anti-ICAM-1, open histograms: staining with the isotype-matched Ig.

**TABLE 2**

Effect of Anti-LFA-1, Anti-ICAM-1, and EDTA on Anti-MHC-II-Induced Cell Adhesion

| Agents tested | BJAB | Ramos |
|---|---|---|
| Medium | +++ | ++ |
| anti-LFA-1 (10 $\mu$g/ml) | +++ | ++ |
| anti-ICAM-1 (10 $\mu$g/ml) | +++ | ++ |
| anti-LFA-1 + anti-ICAM-1 | +++ | ++ |
| EDTA (10 mM) | +++ | ++ |

*Note.* Cells resuspended at a cell density of $1 \times 10^6$/ml in RPMI 1640 medium supplemented with 10% FCS were incubated for 18 hrs with 1 $\mu$g/ml of anti-MHC-II mAbs in the presence or absence of the agents tested at the concentrations indicated, and cell adhesion was determined microscopically as described in Materials and Methods. Results are representative of three separate experiments.

PTK-specific inhibitors, herbimycin A and genistein prevent MHC-II-triggered cell adhesion (2-4,7), indicating that tyrosine phosphorylation may be an early and requisite signal in the response. Furthermore, tyrosine phosphorylation is shown to be one of early event following MHC-II ligation (23-26). In fact, MHC-II ligation stimulates src-type PTKs such as $p55^{fgr}$ and $p53/p56^{lyn}$ in human B cells. In addition, the unique anti-CD45 mAb inhibits MHC-II-triggered cell adhesion by affecting the pattern of tyrosine phosphorylation (8). Thus, tyrosine phosphorylation plays an important role in MHC-II-triggered cell adhesion signaling.

Recently, it has been reported that in lymphocytes and other hematopoietic lineage cells, exogenously added (27) or endogenously produced $H_2O_2$ (28,29) stimulate several src-type and other type of PTKs. In addition, the B-cell surface antigen, the TAPA-1 (CD81) is shown to elicit its anti-proliferative effect through the activation of tyrosine phosphorylation pathway that is regulated by intracellular GSH levels (30). In Jurkat T cells, intracellular GSH levels regulate the tumor necrosis factor receptor-triggered tyrosine phosphorylation and the increase in intracellular $Ca^{2+}$ levels (31). Thus, both increasing intracellular ROS levels and decreasing cellular antioxidants such as GSH lead to the modulation of tyrosine phosphorylation. These observations strongly suggest that the cellular redox status is an intracellular regulator of tyrosine phosphorylation. Taken together with our results, there raises an intriguing possibility that the cellular redox status regulates MHC-II-triggered cell adhesion by modulating tyrosine phosphorylation, an important event in the signal transduction. We are currently investigating the possibility.

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