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Abstract

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KEYWORDS: autoimmune hemolytic anemia, phagocytosis, antibody-dependent cell mediated cytotoxicity, monocytes

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RETICULOENDOTHELIAL CELL FUNCTION IN AUTOIMMUNE HEMOLYTIC ANEMIA (AIHA): STUDIES ON THE MECHANISM OF PERIPHERAL MONOCYTE ACTIVATION

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Abstract. We examined the activity of peripheral blood monocytes in patients with autoimmune hemolytic anemia (AIHA) using an *in vitro* assay of monocyte-macrophage interaction with erythrocytes and an antibody-dependent cell-mediated cytotoxicity (ADCC) assay. The monocytes of AIHA patients in the hemolyzing period phagocytized autologous sensitized red cells and anti-D coated red cells more avidly than normal control monocytes. There was no significant relationship between phagocytic activity and ADCC activity. The activated monocytes phagocytized autologous sensitized red cells, but had no ADCC activity in a short time ^{51}Cr release assay. Phagocytic activity of the patients' monocytes against autologous erythrocytes rapidly decreased after treatment with prednisolone even though the red cell sensitization with antibody remained almost the same as during the hemolyzing period. We postulated that the activation of monocytes in AIHA was due to the "arming" effect of anti-erythrocyte antibody, but we think that other mechanisms may also be involved in the activation of monocytes.

Key words : autoimmune hemolytic anemia, phagocytosis, antibody-dependent cell mediated cytotoxicity, monocytes.

It is well known that in warm reactive autoimmune hemolytic anemia (AIHA), various anti-erythrocyte antibodies are produced and antibody-coated red cells are destroyed in the reticuloendothelial cell system. Several investigators have reported that the reticuloendothelial cell function in AIHA was activated by an unknown mechanism and that severe hemolysis developed even though red cells were sensitized with small amounts of antibody (1-3). MacKenzie (1) suggested that some unknown serum factor (s) might play an important role in peripheral monocyte activation in AIHA. In the present paper, we report the results of experiments using human monocytes, which are representative reticuloendothelial cells. Peripheral monocytes of AIHA patients were shown by ADCC and phagocytosis assay to be activated. The IgG antibodies eluted from red cells of patients with AIHA confered on normal monocytes the ability to bind and phagocytize non-sensitized erythrocytes and enhanced the phagocytosis of sensitized erythrocytes. We propose that one mechanism of monocyte activation might be the sensitization of monocytes by anti-erythrocyte antibodies. This mechanism is similar

to that of the "arming" of macrophages by cytophilic antibodies previously described by Boyden (4).

MATERIALS AND METHODS

Buffers and reagents. Hanks' balanced salt solution (HBSS) and bovine serum albumin (BSA) were purchased from Gibco Labo. The medium used was medium 199 (Gibco Labo) supplemented with 10 % fetal calf serum (FCS), penicillin (10,000 iu/dl) and streptomycin (10 mg/dl). Macrophage separating plates (60 mm × 15 mm; MSP) and MSP-E (EDTA and FCS added to PBS) were obtained from Japan Immune Labo. Anti-D serum was from Dade Diagnostic Co., Ltd.

Patient. A diagnosis of AIHA was made in patient who had anemia, reticulocytosis, a positive direct antiglobulin test (DAT) and no evidence of bleeding. Clinical features and serologic data for three patients on admission are summerized in Table 1. All of them had a positive-DAT due to IgG sensitization.

TABLE 1. HEMATOLOGICAL AND SEROLOGICAL FEATURES OF THREE PATIENTS WITH AIHA

	Patient (1)	Patient (2)	Patient (3)
Age/Sex	46/F	75/F	73/M
RBC, $\times 10^4/mm^3$	204	202	188
Hb, g/dl	7.4	7.2	7.4
Ht, %	22.1	21.4	22.6
Reticulocyte, %	20.6	7.2	22.0
Thrombocyte, $\times 10^4/mm^3$	45.2	21.4	36.6
T. Bil, mg/dl	1.6	0.94	3.47
D. Bil, mg/dl	0.75	0.43	0.48
LDH, IU/l	666	172	993
GOT, IU/l	14	15	60
Haptoglobin, mg/dl	10	45	N.D
DAT using			
Anti-whole serum	++	++	+++
Anti-IgG	+	+	++
Anti-IgA	+	+	−
Anti-IgM	−	−	−
Anti-C3	+	−	+++
Indirect Coombs'	−	−	−

RBC: Red blood cell, Hb: Hemoglobin, Ht: Hematocrit, T. Bil: Total bilirubin, D. Bil: Direct bilirubin, LDH: Lactate dehydrogenase, DAT: Direct antiglobulin test, IU: International unit, GOT: Glutamic oxaloacetic transaminase.

Target red cells. Heparinized venous blood from a patient with AIHA and a healthy doner with type O, Rh-positive blood group was collected under sterile conditions, washed 4 times with isotonic saline and adjusted to a concentration of 5 % (vol/vol) in HBSS. In addition, one volume of a red cell suspension from a healthy donor was incubated for 30 min at 37 ℃ with the same volume of appropriately diluted anti-D serum. After incubation, the cells were washed 4 times and suspended in HBSS at a concentration of 5 %.

Chromium labelling of the red cells. Each 0.1 ml aliquot of the 5 % sensitized red cell suspensions (2×10^8 cells/ml) was incubated with 100 μCi sodium chromate for 120 min at 37 ℃ in a water bath. Cells were washed 4 times, resuspended in medium, and adjusted to a concentration of 2×10^5 cells/ml. Radioactivity was measured with a gamma emission spectrometer.

Elution of red cell antibodies. Eluates were prepared using the modified method of Weiner (5). In brief, the sensitized red cells were washed 3 times with saline, tightly packed and the supernatant carefully removed. The tube was exposed to a temperature of between −6 ℃ and −35 ℃ until the cells were laked. They were then thawed at room temperature, and ten volumes of chilled (−6 ℃) 50 % ethanol was added to them. The tube was rapidly inverted and the alcohol thoroughly mixed with the laked cells. The cells were immediately frozen and kept for 60 min at −20 ℃ to precipitate the antibody proteins. The tube was then centrifuged at 3000 r.p.m for at least 5 min. The supernatant was removed, and the tube was filled with distilled water. The tube was then shaken by hand to mix the sediment thoroughly with the distilled water, and re-centrifuged. The supernatant was again removed, and 6 % BSA was added to the sediment and thoroughly mixed. The tube was then incubated at 37 ℃ for 30 to 60 min. After incubation, the tube was centrifuged and the supernatant, which contained the antibody eluate, was transferred to a clean tube for future use. The antibody activity of the eluate obtained by this method did not decrease for at least 3 months if it was stored at −20 ℃ and below.

Preparation of purified monocyte suspension. Both autologous and allogeneic monocytes were isolated from heparinized whole blood as previously reported (6). Briefly, the heparinized blood was diluted with an equal volume of saline. Six-milliliter aliquots of diluted blood were layered over 3 ml of Conray-Ficoll 400 in 13 × 105 mm glass tubes. The tubes were then centrifuged at 400 G for 20 min. After centrifugation, the mononuclear cells at the interface were harvested and washed 3 times in saline. After the final wash, the mononuclear cells were suspended in medium and adjusted to a concentration of 2-3 × 10^6 cells/ml. Four-milliliter aliquots of cell suspension were added to MSP-P plates and incubated at 37 ℃ for 60 min in a 5 % CO_2 incubator. After incubation, the plates were washed gently four times with HBSS to remove the non-adherent cells. Then, 3 ml of MSP-E was added to each plate, and the cells were incubated for 30 min at 4 ℃. The adherent cells were harvested by gentle pipetting and washed 3 times with medium. These adherent cell preparations consistently contained monocytes and less than 1 % lymphocytes, as determined by morphologic and non-specific esterase staining.

Antibody-dependent monocyte-mediated ^{51}Cr release assay. ^{51}Cr-release was measured after incubation of labelled antibody-coated red cells with monocytes. Equal volumes (0.5 ml) of an antibody-coated red cell suspension (2×10^5 cells/ml) and purified monocyte suspension (2×10^5 cells/ml) were mixed in 13 × 105 mm glass tubes in triplicate and incubated in a moisturized 5 % CO_2 incubator at 37 ℃ for 4 h. The cell suspensions were centrifuged at 200 G for 3 min prior to incubation. After incubation, each tube was centrifuged at 400 G for 10 min to sediment unlysed cells. Supernatant fluid (0.5 ml) was then carefully removed from each tube, and the radioactivity was determined using a gamma emission spectrometer. Chromium release from non-antibody-coated red cells with effector cells were measured in each experiment. Monocyte-mediated red cell lysis was calculated using the formula: Net % ^{51}Cr release $= \frac{A - B}{C} \times 100$, where A was the mean cpm in the supernatant of tubes containing antibody-coated target plus effectors, B was the mean cpm in the supernatant of tubes containing only antibody-coated targets (without effectors) and C was the mean total cpm of targets added to

each tube. The spontaneous release of ^{51}Cr was always less than 2 %, and release from non-antibody coated targets incubated with effectors did not exceed spontaneous release. Student's t test was used to determine the significance among the tubes.

Quantitation of erythrophagocytosis by monocytes. Mononuclear cells suspended in medium (0.2 ml, 5×10^6 cells/ml) were gently layered onto coverslips (12×32 mm) in Leighton tubes and incubated in a moisturized 5 % CO_2 incubator for 60 min at 37 ℃. After incubation, the coverslips were rinsed 3 times with warm HBSS to remove non-adherent cells, and 0.1 ml of a 0.5 % red cell suspension was added to Leighton tubes. The suspension was incubated for another 60 min under the same conditions. After incubation, the coverslips were washed with warm HBSS, dried, fixed in methanol for 3 min, stained with Giemsa and examined for erythrophagocytosis under a light microscope. Percent phagocytosis was defined as the number of phagocytized monocytes counted, and the phagocytosis index was defined as the number of red cells phagocytosed per 100 monocytes counted. The mean and standard error of triplicate samples were calculated. Student's t test was used to asses the significance of differences between sets of samples.

Sensitization of normal monocytes with anti-RBC antibody (Arming). Anti-RBC antibodies were eluted from red cells of a patient No. 3 and from anti-D coated red cells as described above. Sensitization of normal monocytes with anti-RBC antibodies (arming) was done using the modified method of Boyden (4). Briefly, the eluted anti-RBC antibodies (1.0 ml) were added to the monocyte suspensions in a glass tube (1.0 ml, 5×10^6 cells/ml) or the monocyte monolayer in a Leighton tube and incubated at 4 ℃ for 30 min. After incubation, the eluate was discarded and the monocyte monolayer or suspensions were washed vigorously six times with cold HBSS to remove free antibodies. After washing, these armed monocyte preparations were used in each experiment together with normal control monocytes.

RESULTS

The patients' serologic profiles are shown in Table 1. All three patients had a positive DAT using anti-IgG antiglobulin serum. Table 2 shows the *in vitro* interactions of monocytes with red cells from patient No. 1 and patient No. 2 in the hemolyzing stage. Both patients' monocytes phagocytosed autologous sensitized red cells much more avidly than normal human monocytes. There was a good correlation between the % phagocytosis and the phagocytosis index. Table 3 shows monocyte interactions with anti-D coated red cells. Patients' monocytes were obtained in the hemolyzing stage. Monocytes from both patient No. 1 and

TABLE 2. PHAGOCYTOSIS BY ALLOGENEIC MONOCYTES OF RED CELLS FROM AIHA PATIENTS IN THE HEMOLYZING STAGE

Effector cells	Target cells	Anti-IgG Coombs'	Phagocytosis %	Phagocytosis index
Normal monocyte	Patient (1) red cells	+	5.4 ± 2.2	6.5 ± 3.9
Normal monocyte	Patient (1) red cells	+	8.5 ± 0.7	8.6 ± 1.1
Patient (1) monocyte	Patient (1) red cells	+	26.0 ± 6.0	28.0 ± 5.9
Normal monocyte	Patient (2) red cells	+	2.8 ± 1.4	2.6 ± 1.0
Normal monocyte	Patient (2) red cells	+	1.5 ± 0.7	1.7 ± 0.9
Patient (2) monocyte	Patient (2) red cells	+	11.8 ± 3.1	11.7 ± 2.8

Incubation was carried out for 60 min at 37 ℃.

TABLE 3. *IN VITRO* PHAGOCYTOSIS OF ANTI-D COATED NORMAL HUMAN RED CELLS BY NORMAL AND PATIENT MONOCYTES

Effector cells	Target cells	Phagocytosis %	Phagocytosis index
Normal monocyte	×4 diluted anti-D coated red cells	75.8 ± 3.6	201 ± 36
Normal monocyte		71.3 ± 6.4	181 ± 19
Patient (1) monocyte[a]		71.9 ± 6.5	214 ± 21
Normal monocyte	×20 diluted anti-D coated red cells	74.8 ± 5.2	167 ± 15
Normal monocyte		76.8 ± 1.8	150 ± 19
Patient (2) monocyte[a]		78.8 ± 4.2	207 ± 26
Normal monocyte	×40 diluted anti-D coated red cells	60.0 ± 1.9	115 ± 15
Normal monocyte		58.9 ± 7.9	93 ± 22
Patient (2) monocyte[a]		76.0 ± 7.5	145 ± 23

[a]: Patient monocytes were obtained in a hemolyzing stage

patient No. 2 phagocytosed many more anti-D coated red cells than normal control monocytes if the target red cells were sensitized with highly diluted anti-D antibody (× 20 or × 40 diluted). When the target red cells were strongly sensitized with anti-D antibody (× 4 diluted), there was no difference in phagocytic activity between monocytes from patient No. 1 and normal control monocytes. The phagocytosis index reflected the monocyte activity more sensitively than % phagocytosis.

Next, we compared the ADCC activity between patient monocytes and normal control monocytes. Fig. 1 (a) shows that there was no significant difference between monocytes from patient No. 1 and normal control monocytes in ADCC activity toward both the patient's own red cells and anti-D coated red cells. No ADCC activity was demonstrated by either normal or the patient's monocytes against the patient's own red cells, though significant phagocytosis was observed morphologically (Fig. 1 (a) and Table 2). Monocytes from patient No. 2 as well as normal monocytes also had no ADCC activity against the patient's own sensitized red cells. However, toward anti-D coated red cells, the patient's monocytes showed stronger ADCC activity than normal monocytes (Fig. 1 (b)).

In order to elucidate the serum factor responsible for the monocyte activation in hemolyzing patients, we tried to sensitize (arm) effector cells with antibodies. Tables 4 and 5 show the "arming" effect of eluted anti-RBC antibody toward normal human monocytes. It was shown that armed monocytes phagocytosed non-sensitized and sensitized red cells more avidly than normal monocytes. Armed monocytes obtained ADCC activity against non-sensitized red cells, but no different ADCC activity between armed monocytes and normal monocytes was seen against sensitized red cells (Fig. 2).

Positive DAT and *in vitro* erythrophagocytosis was still observed in non-

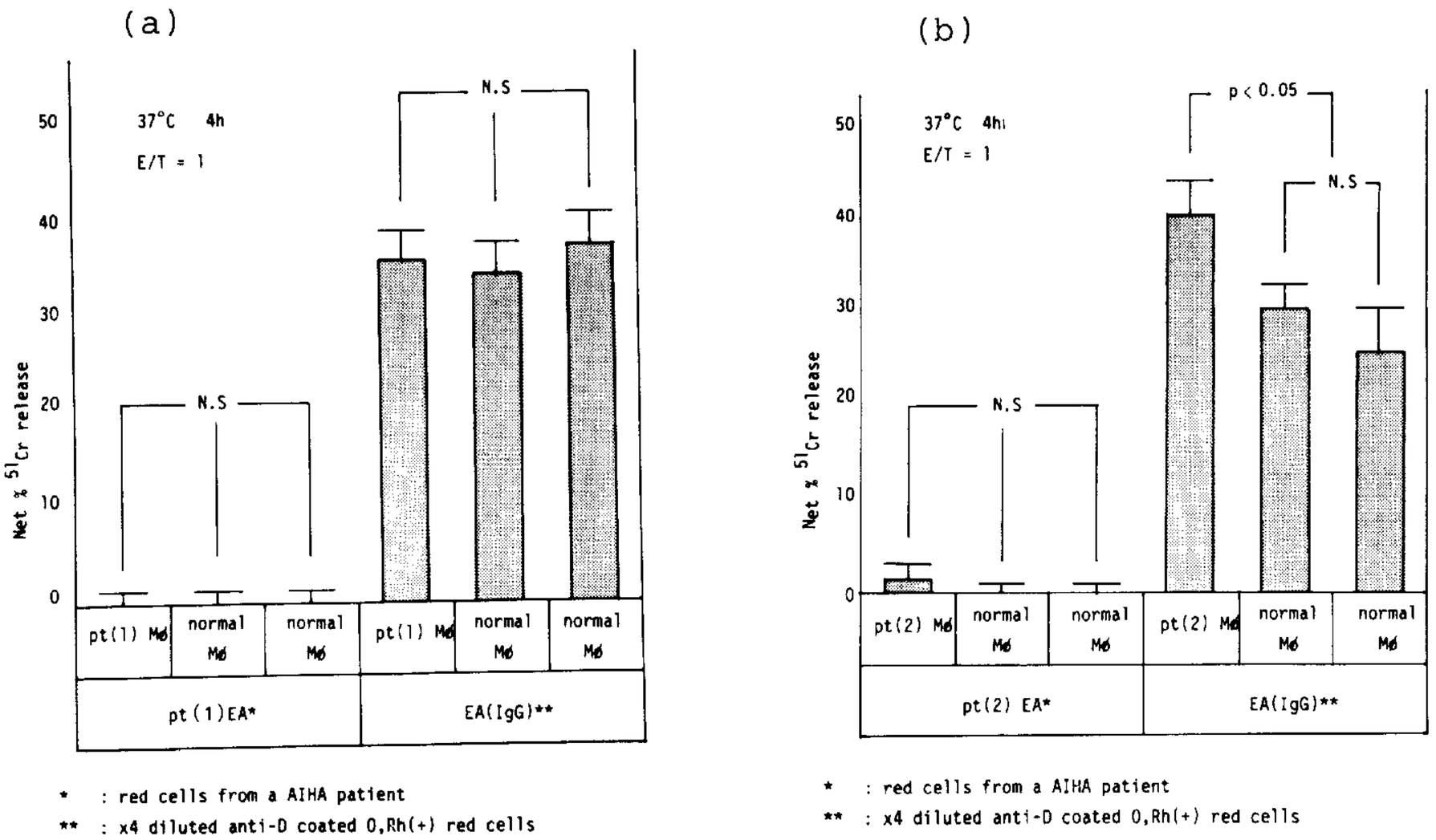


Fig. 1. Comparison of ADCC activity between normal and patients' monocytes. Patients' monocytes and *in vivo* sensitized autologous erythrocytes (EA) were taken when patients were in the active hemolyzing stage. (a) Monocytes and erythrocytes from patient No. 1 and (b) from patient No. 2. Anti-D (× 4 diluted) coated normal type O erythrocytes (EA (IgG)) were also used as a target in each experiment. Incubation was carried out for 4 h at 37℃. The effector/target ratio (E/T) was 1 : 1.

TABLE 4. PHAGOCYTIC ACTIVITY OF NORMAL MONOCYTES AND ARMED MONOCYTES AGAINST SENSITIZED AND NON-SENSITIZED ERYTHROCYTES

Effector cell		Target cell	Phagocytosis index
Normal monocyte	+	EA (IgG[a])	42.8 ± 3.4
Normal monocyte	+	E	0
Armed monocyte[b]	+	E	12.2 ± 1.8
Armed monocyte[b]	+	EA (IgG[a])	70.5 ± 5.4

[a] IIgG anti-RBC antibody eluted from red cells of a patient with AIHA.

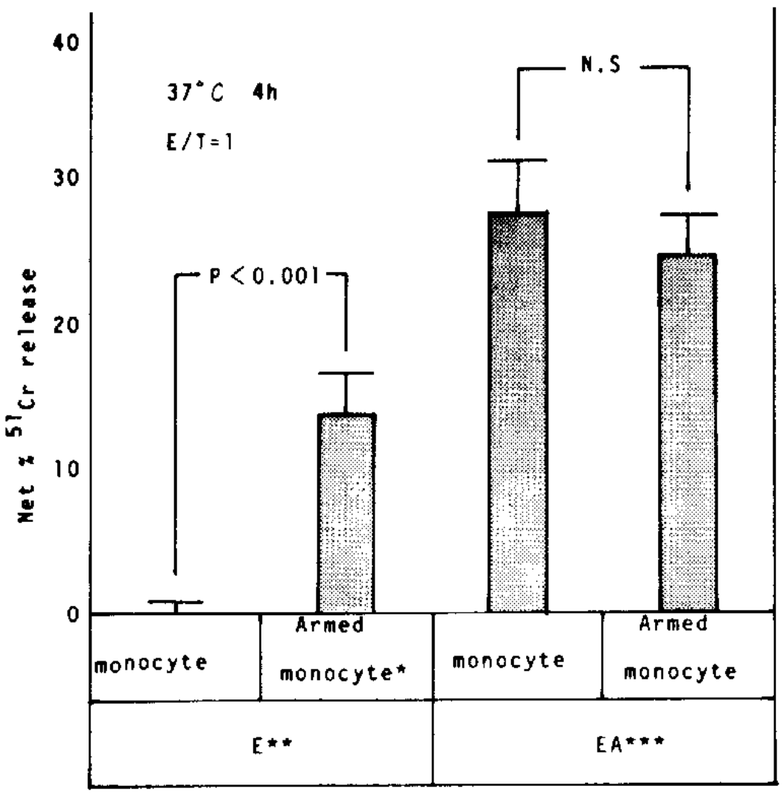
[b] Normal human monocytes preincubated with eluted anti-RBC antibodies at 4℃ for 60 min and washed 6 times with cold HBSS.

TABLE 5. ARMING EFFECT OF ELUTED ANTI-D ANTIBODIES TOWARD NORMAL HUMAN MONOCYTES AS EVALUATED BY PHAGOCYTOSIS OF SENSITIZED AND NON-SENSITIZED ERYTHROCYTES

Effector cell		Target cell	% Phagocytosis	Phagocytosis index
Normal monocyte	+	EA	62.0 + 3.7	103.0 ± 16.0
Normal monocyte	+	E	0	0
Armed monocyte[a]	+	EA	59.2 + 9.6	122.4 ± 16.0
Armed monocyte	+	E	42.4 + 4.6	59.3 ± 12.5

EA: Autologous erythrocytes, Normal human O, Rn (+) red cell.

E: Effector cells, Normal human O, Rh (+) red cell sensitized with anti-D antibody. [a], Normal human monocyte preincubated with eluated anti-D antibody at 4℃ for 60 min and washed 6 times with cold Hank's balanced salt solution (HBSS).



* normal human monocyte preincubated with eluated anti-D antibody at 4°C for 60 min and washed 6 times with cold HBSS

** normal human O^+ red cells

*** x10 diluted anti-D coated human O^+ red cells

Fig. 2. Arming effect of the eluted anti-D antibodies toward normal human monocytes as evaluated by ADCC assay. Normal human type O erythrocytes (E) and anti-D (×10 diluted) coated normal human type O erythrocytes (EA (IgG)) were used as targets.

TABLE 6. HOMOLOGOUS AND AUTOLOGOUS MONOCYTE PHAGOCYTOSIS OF ERYTHROCYTES FROM AIHA PATIENTS IN REMISSION AFTER PREDNISOLONE TREATMENT

Effector cell	Target cell	Anti-IgG Coombs'	Phagocytosis index
Normal monocyte	Patient (1) red cells	+	5.2 ± 2.1
Normal monocyte	Patient (1) red cells	+	4.7 ± 2.2
Patient (1) monocyte	Patient (1) red cells	+	5.7 ± 1.9
Normal monocyte	Patient (2) red cells	+	2.1 ± 0.9
Normal monocyte	Patient (2) red cells	+	1.8 ± 1.0
Patient (2) monocyte	Patient (2) red cells	+	1.7 ± 0.9

hemolyzing patients after prednosolone treatment, but phagocytosis indexes were significantly lowered and no difference in phagocytic activity was observed between the patients' and normal monocytes (Table 6).

DISCUSSION

It is known that peripheral monocytes are activated in various diseases, but there is little data correlating monocyte activity with red cell destruction. MacKenzie found that monocytes from patients with AIHA showed a higher rate of ingestion of IgG sensitized red cells than normal human monocytes (1). Kay and Douglas also studied 21 patients with AIHA and found that 15 of 17 patients showed

enhanced monocyte phagocytic activity against autologous sensitized red cells (2). In our study, monocytes from patients with AIHA in the hemolyzing stage phagocytized autologous red cells and anti-D IgG sensitized red cells more avidly than normal control monocytes (Tables 1 and 2). However, after treatment with prednisolone, the difference in monocyte phagocytic activity between patients and normal control donors disappeared (Table 6). These facts indicate that hemolytic activity in AIHA depends not only on the degree of sensitization of red cells but also on monocyte activity. We assumed that one of the mechanisms responsible for monocyte activation was the sensitization of monocytes by cytophilic anti-erythrocyte antibodies. Cytophilic antibodies are immunoglobulins which become attached to the surface receptors of macrophages and lymphocytes and confer upon these non-specific immune cells the ability to react with specific antigens (4). We presensitized (armed) normal human monocytes with eluted anti-erythrocyte auto-antibodies *in vitro*. These antibodies bound to the monocyte surface via the Fc-receptors and armed these monocytes to become specifically reactive with human erythrocytes. Bakacs and his colleagues reported that non-T cells from individuals sensitized against antigen D were found to lyse type O, Rh-positive erythrocyte (7). They suggested that sensitized donors have lymphocytes armed with specific antibodies *in vivo*. One of the present authors previously reported that the anti-thyroglobulin antibodies in sera of patients with Hashimoto's diseases were cytophilic for human monocytes and rendered normal monocytes cytotoxic against thyroglobulin-coated target cells (8). In our present study, armed monocytes ingested non-sensitized red cells and phagocytosis increased when target red cells were presensitized with anti-erythrocyte antibodies (Tables 4 and 5). We also found that armed monocytes obtained the capability of direct ADCC lysis of non-sensitized red cells (Fig. 2). However, when target red cells were presensitized with anti-erythrocyte antibody, ADCC lysis by armed monocytes did not increase significantly (Fig. 2). This result might be due to the capacity of effector cells for ADCC lysis reaching a maximum without arming.

Munn and Chaplin observed a striking increase in rosette formation of monocyte monolayers from normal subjects during the course of viral infections (9). Atkinson *et al.* showed increased clearance of IgG sensitized red cells in BCG-infected animals, hypothesizing that increased macrophage activation in such animals was responsible for shortened *in vivo* red cell survival (10). We also reported that cytolytic activity of monocytes from patients with inflammatory diseases was enhanced compared to that of normal persons (11). These data suggest that there might be many other nonspecific and specific mechanisms of macrophage activation.

Fries *et al.* demonstrated that monocyte IgG Fc-receptor number was increased in patients with AIHA, and that glucocorticoid administration was associated with a dose-dependent decrease in monocyte IgG Fc-receptor number in normal volunteers and patients (14). Our present results also showed that the enhanced monocyte activity in hemolyzing patients disappeared after prednisolone treatment. It

was suggested that this was due to the direct effect of corticosteroid on monocytes.

Recently, diverse opinions about the effect of corticosteroids on Fc-receptors of monocytes have appeared. Schreiber *et al.* showed that solubilized corticosteroids at concentrations between 10^{-4} and 10^{-3} M inhibited IgG Fc-receptor activity in a dose-dependent fashion (12). In contrast, Kurlander showed that monocytes incubated *in vitro* in medium containing hydrocortisone sodium succinate for 2-3 days bound, ingested and lysed a large number of red cells coated with IgG anti-D (13). We also examined the effect of corticosteroids using methylprednisolone on IgG Fc-receptor-mediated antibody-coated red cell lysis, but brief incubation of monocytes with methylprednisolone had no effect on Fc-receptor-mediated interaction (data not shown). The reason why such different results were obtained is not clear, but the differences might be due to the different kinds of steroids, different incubation time, different amounts of steroids used and the different experimental systems. The effectiveness of steroids on immune hemolysis of AIHA *in vivo* might also be due to the variable metabolic products of steroids.

In the present study, we often encountered the dissociation between phagocytosis and ADCC lysis. Patient monocytes avidly ingested autologous red cells, but ^{51}Cr release by patient monocytes was not be observed in a 4 h short-time assay (Fig. 1). The dissociation between phagocytosis and ADCC lysis by monocytes against antibody-coated red cells might be due to the postphagocytic ^{51}Cr release by monocytes (6) and the very small amount of antibody on the red cells. If red cells are sensitized with very large amounts of anti-erythrocyte antibody, monocytes may ingest many erythrocytes, soon lose the capacity for phagocytosis and lyse red cells extracellulary.

In our and other investigator's data (3), the phagocytosis index reflects the monocyte activity in AIHA more sensitively than ADCC lysis if target red cells are sensitized with very small amounts of antibodies. Therefore, we conclude that the phagocytosis index is a better parameter of reticuloendothelial function in AIHA than the ADCC assay when red cell sensitization with autoantibodies is weak.

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