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Cellular cooperation in lymphocyte activation

II. COOPERATIVE RESPONSE OF HUMAN PERIPHERAL T AND B LYMPHOCYTES TO RABBIT ANTI-HUMAN β_2- MICROGLOBULIN

K. KIN, T. KASAHARA,* Y. ITOH, I. SAKURABAYASHI, T. KAWAI & K. MORITA† *Department of Clinical Pathology,* * *Department of Medical Biology and Parasitology, Jichi Medical School, Minamikawachi-machi, Kawachigun, Tochigi-ken,* 329-04, *and* † *First Department of Internal Medicine, Nihon University School of Medicine, Oyaguchi, Itabashi-ku, Tokyo, Japan.*

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SUMMARY

In the present study we attempted to clarify the effects of anti-β_2-microglobulin (a-β_2m) on lymphocyte activation. Neither a-β_2m IgG fraction nor F(ab')$_2$ had a mitogenic effect on either highly purified T or B lymphocytes alone, while their mitogenic effect was observed when T and B lymphocytes were appropriately reconstituted. When T lymphocytes were reconstituted with mitomycin C (MMC) treated B lymphocytes, a negligible decrease in the response to a-β_2m was observed compared to the response of an untreated mixture to a-β_2m. On the other hand, when B lymphocytes were reconstituted with MMC-treated T lymphocytes, the response was markedly diminished. It was found, moreover, that the response of T lymphocytes separated by a semi-permeable membrane from MMC-treated B lymphocytes was not enhanced, while a mixture of T and MMC-treated B lymphocytes in the same chamber showed a marked response.

These results lead to the conclusion that the cells responding to a-β_2m are mainly T lymphocytes whose response is strongly enhanced by B lymphocytes, and that for the mitogenic effect of a-β_2m direct cell-to-cell interaction between T and B lymphocytes is necessary.

INTRODUCTION

The human histocompatibility antigen is composed of two types of polypeptide chains (Cresswell, Turner & Strominger, 1973; Tanigaki *et al.*, 1974). The larger chain carries the allogenic determinants, whereas the small subunit is identical to β_2-microglobulin (β_2m) which is a low molecular weight protein of 11,800 Daltons (Nakamuro, Tanigaki & Pressman, 1973; Peterson, Rask & Lindblom, 1974; Grey *et al.*, 1973).

Among the effects of anti-β_2-microglobulin (a-β_2m) observed was the ability to stimulate DNA synthesis, as well as to induce synthesis and secretion of immunoglobulins in B lymphocytes (Möller & Persson, 1974; Ringden & Möller, 1975). Thus, a-β_2m has been said to cause, non-specifically, a large number of resting B lymphocytes to proliferate and differentiate into antibody-forming cells. Östberg, Lindblom & Peterson (1976) demonstrated that the most pronounced mitogenic effect of a-β_2m antibody was noted in mixtures containing T lymphocytes rather than B lymphocytes alone.

In the present study we have attempted to clarify the existence of a cooperation between T and B lymphocytes in cell culture with the stimulation of a-β_2m by using highly purified human peripheral T and B lymphocytes.

Correspondence: Dr K. Kin, Department of Clinical Pathology, Jichi Medical School, Minamikawachi-machi, Kawachigun, Tochigi-ken, 329-04, Japan.

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MATERIALS AND METHODS

Purification of T and B lymphocytes. The method used to purify T and B lymphocytes has been described elsewhere (Kin *et al.*, 1979; Kasahara *et al.*, 1979). In brief, leucocyte-rich fractions of human peripheral blood were prepared from remnants used for platelet transfusion. These had been separated from healthy male donors by a blood professor cell separator (Hemonetics Model 30, Hemonetics Co., USA). These fractions were diluted with phosphate buffered saline (PBS, pH 7·4), and phagocytic cells were removed as follows; carbonyl iron (5% w/v, 3–6 μm, Wako Pure Chemical Co., Japan) was added to the cell preparation and stirred at 37°C for 45 min. Iron-powder phagocytized cells were then removed with magnetic force. Lymphocytes were separated by the Ficoll–Hypaque (Pharmacia, Sweden, d = 1·077) sedimentation method. Approximately 3×10^8 cells in 1·0 ml of minimum essential medium (MEM) containing 2% of foetal calf serum (FCS, Flow laboratories, Australia) were then applied to a column in which 1·2 g of sterile nylon fibre (Semidull Nylon Staple, Dupont, Wilmington, Delaware, USA) were packed, and kept at 37°C for 45 min. The cells were then eluted with warm (37°C) FCS-MEM. The procedure was designed so that the cells which passed through the nylon fibre column would be the T cell source, whilst those cells retained in the nylon fibre column would be the B cell source. Virtually no null cells were observed in this B cell source.

E-rosette formation was applied to both cell sources. In brief, a mixture comprising a suspension of 5% sheep red blood cells (SRBC, Japan Biological Materials Centre, Tokyo, Japan) and a lymphocyte suspension was held at 37°C for 15 min. This mixture contained 50% FCS. It was then spun at 200 *g* for 5 min and incubated at 4°C for at least 2 hr. E-rosette-forming cells were separated by the Ficoll–Hypaque sedimentation method. E-rosette formation was routinely repeated twice, with the second incubation period lasting overnight. T lymphocytes were obtained after SRBC were lysed by incubation in 0·01 M Tris-0·75% ammonium chloride (pH 7·4) at 37°C for 10 min. Finally, dead cells were removed from the T lymphocytes by Ficoll–Hypaque sedimentation. B lymphocytes were retrieved from the interface of Ficoll–Hypaque and any monocytes remaining amongst the B lymphocytes were removed as completely as possible by adhesion to plastic dishes.

The purity of the T and B lymphocytes was tested by using the E-rosette-forming test and immunofluorescence study for lymphocyte surface immunoglobulins. The purity of the T and B lymphocytes was more than 98%, respectively. The initial lymphocyte number before T and B lymphocyte separation was $2\text{–}3\times10^9$ cells, and the recovery of T and B lymphocytes was 20–30% and 4–6%, respectively.

Lymphocyte culture. Cells were adjusted to 5×10^5 cells/ml in culture medium (RPMI 1640, Nissui Seiyaku, Japan) containing 6% FCS, and incubated in glass tubes (12 × 105 mm) for 72 hr in 5% CO_2, 95% air. 0·5 μCi of tritiated thymidine (^{3}H-TdR, 5·0 Ci/mmol, Japan Isotope Association) was added 16 hr before cell harvesting. Cells were then harvested onto glass fibre filters using a semi-automatic sample harvester (Labo Mash, Laboscience, Japan). The filters were dried and added to 5·0 ml of toluene scintillator and counted in a Packard liquid scintillation spectrophotometer. ^{3}H-TdR incorporation was expressed as the mean count per min (ct/min ± s.d.), and the stimulation index was calculated as the ratio of ^{3}H-TdR incorporation in the presence of a-β_2m to that in the control where lymphocytes only were cultured.

Mitogens. As positive controls, phytohaemagglutinin (PHA, 10 μg/ml, Difco), concanavalin A (Con A, 5·0 μg/ml, Boehringer–Mannheim), pokeweed mitogen (PWM, 10 μg/ml, Gibco), and *Staphylococcus aureus* Cowan I organisms (SpA, 0·01% v/v, provided by Dr T. Matsuhashi, Institute of Medical Science, University of Tokyo) known to be B cell mitogen (Forsgren, Svedjelund & Wigzell, 1976; Kin *et al.*, 1978), were used.

Mitomycin C treatment. Cells (2×10^7/ml) in FCS-MEM were incubated with 50 μg of mitomycin C (MMC; Sankyo Co., Japan) at 37°C for 30 min. The cells were then washed four times and resuspended at 5×10^5 cell/ml in culture medium.

Preparation of a pepsin-digested fragment of a-β_2m IgG fraction. Rabbit anti-human β_2m IgG fraction used was obtained from DAKO-Immunoglobulins Ltd. (Copenhagen, Denmark; lot number 046). 0·7 g of the IgG fraction was mixed with 14 mg of crystallized pepsin in 0·01 M sodium acetate buffer (pH 4·5) and allowed to react for 20 hr at 37°C (Mandy & Nisonoff, 1963). The mixture was adjusted to pH 8·0 to inactivate the pepsin and dialysed in cold 0·2 M borate buffer (pH 8·0). The pepsin-digested fragment was separated by Sephadex G150 (40–120 μm) column chromatography (25 × 100 cm, 0·2 M borate buffer pH 8·0 with 0·01 M sodium chloride). The second highest fraction was collected. After concentration, the antigenicity of the F(ab′)$_2$ of the IgG fraction was determined by immunoelectrophoresis using goat anti-rabbit IgG antiserum, and specific antibody activity was confirmed by purified β_2m obtained from the urine of patients with renal tubular disorders. The molecular weight of the F(ab′)$_2$ fraction was estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Weber & Osborn, 1969). The protein concentration was measured by the method of Lowry *et al.* (1951).

RESULTS

Effect of a-β_2m on purified T and B lymphocytes

The mitogenic activity of a-β_2m at various dilutions was investigated on unfractionated, purified T and B lymphocytes obtained from human peripheral blood. The proliferative activity of each, as measured by the incorporation of ^{3}H-TdR, is shown in Table 1. The final concentration of 1:10 diluted a-β_2m IgG fraction was adjusted to 1·0 mg/ml. In unfractionated lymphocytes, ^{3}H-TdR incorporation was increased by the addition of 1:50 diluted a-β_2m. Furthermore, lymphocytes were markedly activated when 1:10

TABLE 1. Effects of a-β_2m IgG fraction and various mitogens on purified human peripheral T and B lymphocytes

Mitogens	^{3}H-thymidine uptake (ct/min ± s.d.)					
	Unfractionated lymphocytes	SI*	Purified T	SI	Purified B	SI
Control	2057 ± 160		328 ± 45		2274 ± 189	
a-β_2m† 1:1000	1320 ± 196	0·64	286 ± 36	0·88	2259 ± 166	0·99
1:100	1584 ± 167	0·77	303 ± 34	0·92	2312 ± 164	1·03
1:50	9336 ± 1020	4·58	562 ± 47	1·41	2016 ± 172	0·89
1:10	32 854 ± 1650	15·97	816 ± 98	2·49	1830 ± 186	0·81
PHA (10 μg/ml)	70,121 ± 3539	32·09	19,338 ± 1592	58·96	2065 ± 203	0·91
Con A (5 μg/ml)	69,251 ± 3364	33·67	8082 ± 629	24·64	1805 ± 182	0·79
PWM (10 μg/ml)	38,556 ± 2182	18·74	9173 ± 856	27·97	1984 ± 169	0·87
SpA‡ (0·01%)	13,845 ± 1251	6·73	336 ± 25	1·02	52,801 ± 4736	23·22

The cell cultures were initiated with 5×10^5 cell/ml. 1·0 ml of lymphocyte suspensions prepared as described in the Materials and Methods section were incubated for 72 hr in glass tubes. 0·5 μCi of ^{3}H-TdR was added 16 hr before cell harvesting. All cell cultures were performed in duplicate.

* SI = Stimulation index, expressed as the ratio between ^{3}H-TdR uptake with and without addition of a-β_2m or mitogen.

† The protein concentration of a-β_2m IgG fraction was adjusted to 10 mg/ml in PBS, and the final concentration in cell culture was 1·0 mg/ml (1:10 dilution).

‡ *Staphylococcus aureus* Cowan I organisms.

diluted a-β_2m was added. Normal rabbit IgG, rabbit anti-human κ and anti-λ light chain IgG fractions (DAKO-Immunoglobulins Ltd., lot number 066) did not stimulate the lymphocytes at all. In the control, where only the purified lymphocytes were cultured, ^{3}H-TdR uptake by B lymphocytes was significantly higher than that by T lymphocytes. T lymphocytes were only slightly activated by a-β_2m at a 1:10 dilution, but the level of ^{3}H-TdR uptake was significantly lower than that of the B lymphocyte control. At a a-β_2m concentration below 1:50, ^{3}H-TdR uptake was unchanged. Similarly, in B lymphocytes no stimulatory effects of a-β_2m were observed; conversely, at the a-β_2m concentration of 1:10, ^{3}H-TdR uptake tended to be even lower than its control. Cell viability was also examined by using Trypan-blue dye (0·2%) staining, but the cells cultured with a-β_2m were as viable as those in the control. No stimulatory effects were observed in B lymphocytes with PHA, Con A or PWM, while T lymphocytes were activated with these mitogens. B lymphocytes were markedly activated with SpA, while SpA had no effect on T lymphocytes.

These results suggest, therefore, that a-β_2m has mitogenic activity in neither highly purified T nor B lymphocytes alone.

Demonstration of T–B cooperation in the reconstituted culture

As shown in Fig. 1, the highest responsiveness to a-β_2m was observed with the mixture containing both T and B lymphocytes (T/B ratio ranged between 80/20 and 40/60). Marked stimulation was also observed in the B lymphocyte-predominant culture (T/B ratio 20/80). It was clearly shown that purified T and B lymphocytes respond cooperatively to a-β_2m. No significant stimulatory effects of anti-κ or anti-λ IgG fraction were observed on the reconstituted lymphocytes. The next step was to determine which cells respond to a-β_2m. In this experiment a-β_2m F(ab′)$_2$ was used in order to eliminate the effect of the Fc portion. Cells responding to a-β_2m were determined in the reconstituted culture of T and B lymphocytes, when either was previously treated with mitomycin C (MMC). When T lymphocytes were reconstituted with MMC-treated B lymphocytes, the response to a-β_2m was slightly diminished compared to that of the untreated mixture (Fig. 2). On the other hand, when B lymphocytes were reconstituted with MMC-treated T lymphocytes, the response was markedly decreased. In order to determine

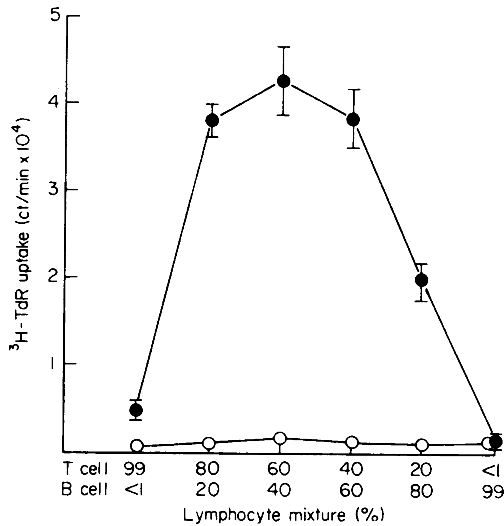

FIG. 1. The effect of a-β_2m IgG fraction on the reconstitution of T and B lymphocytes in cell culture. The cell cultures were initiated with 5×10^5 cells/ml. The final protein concentration of a-β_2m IgG fraction was adjusted to 1·0 mg/ml. (●) a-β_2m, (○) control.

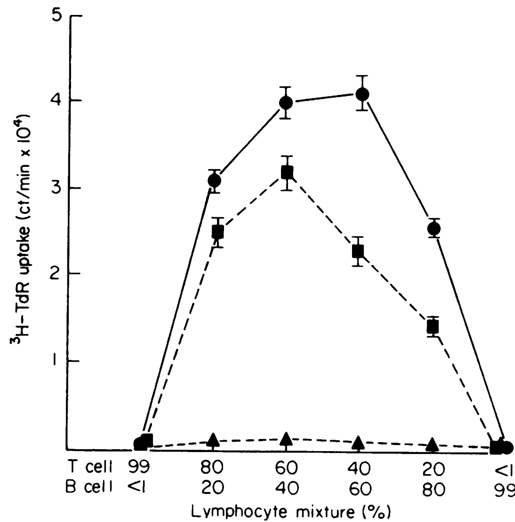

FIG. 2. The effect of a-β_2m F(ab′)$_2$ on the reconstitution of T and B lymphocytes in cell culture. The cell cultures were initiated with 5×10^5 cells/ml. The final protein concentration of a-β_2m F(ab′)$_2$ was adjusted to 1·0 mg/ml. (■) T and mitomycin-treated B lymphocytes; (▲) mitomycin-treated T and B lymphocytes; (●) untreated.

whether direct contact between T and B lymphocytes is necessary, a Marbrook-type culture vessel (Marbrook, 1967; Kasahara *et al.*, 1979), in which two culture chambers were separated by a Millipore filter, was used. It was found that the response of T lymphocytes separated by a semi-permeable membrane from MMC-treated B lymphocytes was not enhanced, while a mixture of T and MMC-treated B lymphocytes in the same chamber showed a marked response (Table 2). The finding indicates that those cells responding to a-β_2m are mainly T lymphocytes, and that B lymphocytes strongly enhance the T cell response to a-β_2m. Further, it was clearly demonstrated that no proliferative response was required to elicit the B cell enhancing effect and that this effect was mediated by direct cell-to-cell interaction.

TABLE 2. Requirement of direct cell-to-cell interaction for the T cell enhancement by B cells

Reconstitution*				
Upper	Lower	Control	a-β_2m 1:10	Ratio†
None	T+Tmmc‡	271±40	796±41	1·0
None	T+Bmmc	377±108	18,570±580	23·3
Tmmc	T	322±93	481±260	0·6
Bmmc	T	267±119	442±31	0·6

* Cells were incubated in the Marbrook-type culture vessel in which two compartments were separated by Millipore filter (0·45 μm). The upper chamber contained 1·0 ml of medium and the lower contained 3·0 ml. After the cells were incubated for 60 hr in the Marbrook culture vessel, 0·5 ml of lower cells were placed in culture tubes, in triplicate, and ^{3}H-TdR incorporation was assayed for 16 hr.

† The ratio to the ct/min of (T+TMMC) in the presence of a-β_2m (1:10).

‡ The proportion of responding T cells ($1·5\times10^6$ cells/3·0 ml) to MMC-treated cells was 3:1.

To confirm their enhancing capacity, B lymphocytes were further separated into non-adherent and adherent cells by the use of plastic dishes. As shown in Fig. 3, both of the adherent and non-adherent B populations, as well as the adherent cells directly separated from peripheral blood, which contained 50–60% of monocytes, could enhance the T cell response to a-β_2m. Among them, the strongest activity was seen in the non-adherent B lymphocytes and only a small percentage of such cells could significantly enhance the T cell response to a-β_2m.

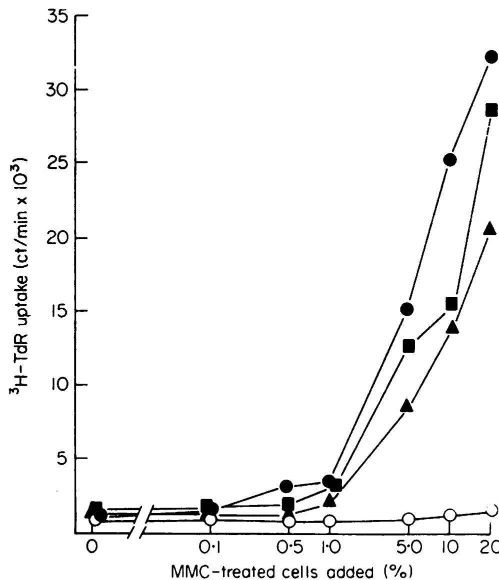

FIG. 3. The effect of the addition of mitomycin-treated cells on the response of peripheral T cells to a-β_2m. MMC-treated cells were added to responding T cells (3×10^5/ml) to constitute 0·1 to 20%. (■——■) MMC-treated adherent population of purified B cells; (●——●) MMC-treated non-adherent population of purified B cells; (▲——▲) MMC-treated adherent cells to plastic dishes directly obtained from unfractioned peripheral blood; (○——○) TMMC.

DISCUSSION

It is well-known that a-β_2m can be mitogenic for human lymphocytes (Ringden & Johansson, 1977). In earlier studies, Möller & Persson (1974) demonstrated that a-β_2m stimulated murine spleen B cells, but not T cells, and induced intracellular immunoglobulin synthesis in blast cells. It was, moreover, shown that both a-β_2m IgG and F(ab')$_2$ could be mitogenic for human peripheral, tonsil and spleen lymphocytes, and that they induced immunoglobulin synthesis (Ringden & Möller, 1975). From these results it was concluded that a-β_2m could serve as a functional marker for B lymphocytes. However, in their experiments, the effect of a-β_2m on purified human T and B lymphocytes was not examined. Recently, Östberg *et al.* (1976) showed that the most pronounced mitogenic effect of a-β_2m was noted in a mixture containing 25–80% T lymphocytes, rather than B lymphocytes alone, suggesting that the B lymphocyte response to a-β_2m would be mediated by T lymphocytes. Our experiments indicate, however, that those cells responding to a-β_2m are predominantly T lymphocytes and their responsiveness is greatly enhanced by the helper effect of B lymphocytes. When cells cultured for 3 days were examined by E-rosette formation, most of blast-transformed cells formed E-rosettes (data not shown). This finding also supports the fact that the responding cells to a-β_2m are T lymphocytes.

Although no significant B cell response was observed within the 3-day culture, in which T and B lymphocytes were reconstituted evenly, immunoglobulin-producing cells were observed by means of cytoplasmic immunofluorescence staining, when cultured for 6–7 days (data not shown). It is therefore supposed that a-β_2m-induced immunoglobulin synthesis by B lymphocytes will require initial T cell activation and its helper effects. This is also supported by the fact that when only B lymphocytes are cultured in the presence of a-β_2m, immunoglobulin production does not occur even in a long-term culture (data not shown).

For the helper effect of B lymphocytes on the T cell response, a B cell proliferative response was not found to be essential, since MMC-treatment is known to inhibit cell proliferation. The enhancing effect of B cells on the T cell response is considered to be mediated by direct T–B cell interaction, because T cell responses are not observed when T and B lymphocytes are separated by a semi-permeable membrane in cell culture, and are only observed when T and B lymphocytes are reconstituted in the same chamber. It has been considered that an enhancing effect on the T cell response is mainly caused by adherent cells (Delespesse *et al.*, 1976; Schmidtke & Hatfield, 1976), but in the present study, the participation of monocytes can be excluded since both phagocytic cells and adherent cells had been almost completely removed. (No phagocytic cells were in fact observed in the cultured cells, data not shown.) Moreover, in this study the effect of the exogenous addition of monocytes was also examined. It was demonstrated, however, that the helper effects of a monocyte-rich fraction were no greater than those of non-adherent B lymphocytes.

The T–B cooperation has been explained as being mainly ascribed to the enhancing effect mediated by T lymphocytes on the B lymphocyte response to various mitogens, such as PWM, Con A and Protein A from *Staphylococcus aureus* (Janossy *et al.*, 1977; Romagnani *et al.*, 1977; Kasahara *et al.*, 1978).

In the present study we have shown that a marked stimulation by a-β_2m is observed in either a T or B lymphocyte predominant culture (T/B ratio, 20/80 or 80/20). Thus, incomplete separation of T and B lymphocytes could possibly lead to the false conclusion being made that a-β_2m can stimulate B lymphocytes.

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