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Immunology

Activation of T and B lymphocytes *in vitro:* Presence of β_2-microglobulin determinants on allogeneic effect factor

(histocompatibility molecules/thymus-derived and bone marrow-derived lymphocytes)

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ABSTRACT **The biologically active entity termed allogeneic effect factor, which is produced by alloantigen-activated T cells and has been shown to regulate triggering and differentiation of B lymphocytes *in vitro,* has previously been demonstrated to possess antigenic determinants coded by genes in the *I* region of the *H-2* gene complex of the mouse. The studies presented here provide evidence that allogeneic effect factor also possesses antigenic determinants identical or cross-reactive with β_2-microglobulin. These observations suggest an important role for β_2-microglobulin in the mechanism of cell–cell interactions and the consequences of such interactions on lymphocyte triggering and differentiation.**

For the past 7–8 years, the phenomena of cell interactions in development and regulation of immune responses have been extensively investigated. During this time, much has been learned about the importance of such cell interactions in regulating the immune system, and although not yet precisely delineated, about the mechanisms by which these interactions take place. In recent years, much evidence has been obtained to indicate a very important role for gene products of the major histocompatibility complex (MHC) in controlling interactions between T (thymus-derived) and B (bone marrow-derived) lymphocytes (1–4) and between macrophages and lymphocytes (5, 6), resulting in the hypothesis that there are cell interaction (*CI*) genes located in MHC that code for the molecules responsible for mediating cell–cell interactions (2, 3, 7–9). This notion has been strengthened by the recent demonstrations by ourselves (10–13) and by others (14, 15) that certain biologically active T cell factors derived from antigen-activated T cells bear determinants of gene products known to map in the MHC of the mouse, and more specifically in the *I* region of *H-2*.

Amid extensive speculation on the probable importance and involvement of soluble T cell factors in T–B cell interactions (16–18), considerable attention has been focused on the identification and characterization of such factors. In previous reports from our own laboratory, we have described the biological and biochemical properties of such a factor, which appears to induce and regulate triggering and differentiation of other lymphocytes (9–13). This factor is obtained from culture supernatants of short-term *in vitro* mixed lymphocyte reactions between alloantigen-activated T cells and the appropriate target cell population; we have termed the active moiety of such supernatants allogeneic effect factor (AEF) (10). The predominant biological feature of AEF that has been studied in depth is its capacity to facilitate triggering and differentiation of B lymphocytes to develop antibody responses to antigen-stimulation *in vitro* in the absence of intact T cell function; this effect is not antigen-specific (10). Analyses of the physicochemical and immunochemical properties of AEF have revealed that: (*1*) it is a protein of molecular size around 30,000–45,000 daltons; (*2*) it is not immunoglobulin in nature; and (3) it possesses antigenic determinants coded for by genes in the *I* region of the *H-2* complex; thus, the biological activity of AEF can be absorbed by an immunoadsorbent prepared from antisera directed against *I* region-associated (Ia) antigens (10–13).

In view of the interesting association demonstrated in recent years between the ubiquitous small protein β_2-microglobulin (β_2m) and various gene products of the MHC in several species (see refs. 19 and 20 for reviews), and the clear evidence for the presence of histocompatibility antigen determinants on AEF and certain other active T cell factors, we sought to ascertain whether or not an association existed between β_2m and the biological and/or structural properties of AEF. The studies presented in this paper demonstrate conclusively the association between an entity identical to, or cross-reactive with, β_2-microglobulin and the biologically active moiety of AEF, since the latter can be completely and specifically absorbed by antisera directed against β_2m determinants.

MATERIALS AND METHODS

The tissue culture system, the assay for *in vitro* antibody responses to sheep erythrocytes (SRBC), the preparation of anti-θ serum, and the method used for depletion of T lymphocytes have been described in detail elsewhere (10, 21, 22). Inbred DBA/2 (*H-2*^{d}) mice and (C3H × DBA/2)F_1 hybrids (*H-2*$^{k/d}$) were obtained from The Jackson Laboratories, Bar Harbor, Me.

Preparation of Allogeneic Effect Factor (AEF). The details of AEF production have been described previously (10). Briefly, DBA/2 thymocytes were activated *in vivo* against (C3H × DBA/2)F_1 target lymphoid cells in adoptive DBA/2 hosts for 7 days. The activated T lymphocytes were obtained from the spleens of recipients and co-cultured with irradiated (C3H × DBA/2)F_1 lymphoid cells *in vitro* for 24 hr in either medium containing fetal calf serum as described earlier (10) or more recently in serum-free medium, using a procedure to be reported shortly‡. The culture supernatants containing AEF were collected by centrifugation, filtered through 0.45 μm Millipore filters (Millipore Corp., Bedford, Mass.), and stored at −20°.

Abbreviations: AEF, allogeneic effect factor; β_2m, β_2-microglobulin; B cell, bone marrow-derived; *CI* genes or molecules, cell interaction genes or molecules; Ia, *I* region-associated antigens; Ig, immunoglobulin; MHC, major histocompatibility complex; PFC, plaque-forming cells; SRBC, sheep erythrocytes; T cell, thymus-derived.

‡ D. Armerding, Z. Eshhar, and D. H. Katz, manuscript in preparation.

Antisera and Preparation of Immunoadsorbents. Human β_2m was purified from the urine of kidney transplant patients as previously described (23). The mouse analogue of β_2m was isolated from the culture fluid supernatants of the DBA/2 mouse tumor line L5178Y, by gel filtration, ion-exchange chromatography, and affinity chromatography[§]. This preparation was characterized as having β_2m determinants by virtue of its capacity to inhibit the binding of ^{125}I-labeled human β_2m to anti-β_2m in a radioimmunoassay. The purity of this preparation, however, has not been assessed as yet due to insufficient quantities being available for this purpose. Antisera to human and mouse β_2m were prepared in rabbits and tested for serological activity as described previously (23, 24, §). The following antisera were employed: (*1*) anti-human β_2m antisera which were not cross-reactive with mouse β_2m; in one instance, the serum was absorbed with purified human β_2m to remove all specific antibodies; (2) anti-human β_2m antiserum which cross-reacts with the mouse β_2m as assessed by inhibition of the radioimmunoassay; (3) antiserum raised in rabbits initially immunized with human β_2m and then boosted with mouse β_2m; (*4*) anti-mouse β_2m antiserum; (5) normal rabbit serum; and (*6*) a polyvalent anti-mouse immunoglobulin (Ig) antiserum prepared as described previously (25).

Immunoadsorbents were prepared by the method of Avrameas and Ternyck (26) by direct crosslinking of immune or normal sera with glutaraldehyde at pH 5.0. Unactionated AEF was absorbed directly with excess immunoadsorbents in a batchwise procedure as described previously (11). The absorbed AEF was assayed for biological activity at 1:10 to 1:40 dilutions after filtration through 0.45 μm Millipore filters.

RESULTS

Absorption of AEF activity by anti-β_2m antisera

In the experiment shown in Fig. 1, unprimed DBA/2 spleen cells depleted of T lymphocytes failed to develop an appreciable anti-SRBC plaque-forming cell (PFC) response; untreated control spleen cells developed around 400 PFC of the IgG class in response to SRBC (data not shown). The addition of unabsorbed AEF to the T cell-depleted spleen cells reconstituted and facilitated the response in magnitudes proportional to the concentration of AEF used. Three immunoadsorbents prepared from either normal rabbit serum or two anti-human β_2m antisera, one of which had been previously absorbed with purified human β_2m, served as negative controls in this experiment; the latter two sera were deliberately chosen for their incapability to cross-react with mouse β_2m. As shown in Fig. 1, absorption of AEF with these adsorbents did not result in a significant loss of biological activity when compared to unabsorbed AEF. However, quite in contrast is the binding capacity of two other crosslinked antisera, one of which was raised in rabbits against the presumed mouse β_2m, the other was obtained by priming with human β_2m and boosting with mouse β_2m. Tested at a dilution of 1:40, the activity of AEF was completely abolished. At the highest concentration, the absorbed AEF triggered responses of 5 and 18% of that obtained with AEF subjected to absorption on the normal rabbit serum immunoadsorbent.

The results of the preceding experiment demonstrate the capacity of antisera directed against a mouse β_2m-like protein to specifically absorb the biological activity of AEF. Since there is as yet no conclusive proof that these antisera are indeed specific for the mouse β_2m analogue, an attempt was made to corroborate this observation with antiserum prepared against purified human β_2m and which was known to be cross-reactive with the mouse β_2m-like protein. A representative experiment is summarized in Table 1. The IgM response of untreated DBA/2 spleen cells (culture I) was completely abrogated by prior treatment with anti-θ serum plus complement (culture II), and was completely reconstituted by incorporation of unabsorbed AEF into the culture (culture III). An immunoadsorbent prepared with normal rabbit serum failed to remove any biological activity from AEF (culture IV), whereas immunoadsorbents consisting of either anti-mouse β_2m or cross-reacting anti-human β_2m removed more than 80% of the biological activity (cultures V and VI). Most important, however, was the observation that addition of purified human β_2m (100 μg/ml) to the cross-reacting anti-human β_2m immunoadsorbent prior to absorption of AEF substantially blocked the capacity of this adsorbent to remove AEF activity (culture VII). This finding corroborates the observations shown in Fig. 1 and, moreover, demonstrates that the anti-β_2m binding sites in the cross-re-

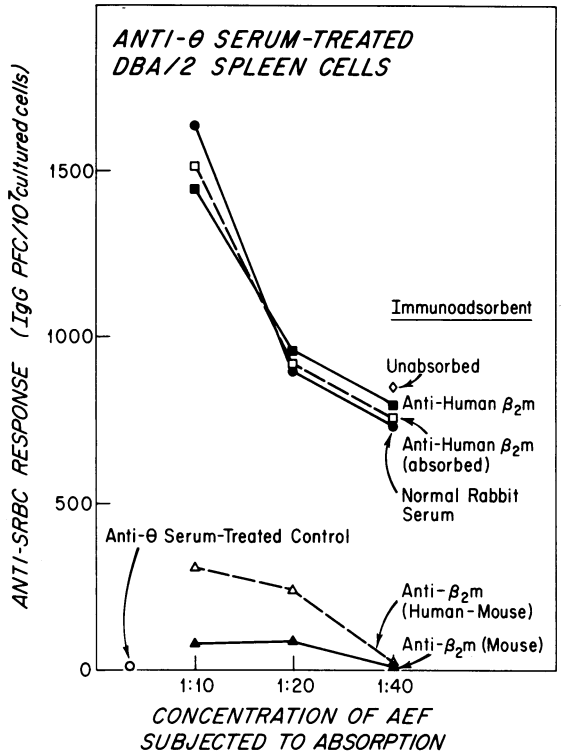

FIG. 1. Absorption of the biological activity of AEF by anti-β_2m antisera. Three different concentrations of AEF were subjected to immunoadsorbents prepared from either: (*1*) normal rabbit serum; (2) rabbit anti-human β_2m antiserum (unabsorbed); (*3*) rabbit anti-human β_2m antiserum (previously absorbed with purified human β_2m); (*4*) rabbit antiserum raised initially against human β_2m and then boosted with mouse β_2m; and (*5*) rabbit anti-mouse β_2m antiserum. These AEF were then tested and compared to unabsorbed AEF for activity on the *in vitro* response to SRBC or anti-θ serum-treated spleen cells. The control response of anti-θ-treated cells in the absence of AEF is shown at the lower left. The activity of unabsorbed AEF is shown at only one dilution (1:40) for purposes of clarity of the figure but paralleled the normal rabbit serum-absorbed AEF at the higher concentrations. The IgG antibody responses are presented and paralleled the IgM response pattern of these same cultures (not shown).

§ R. T. Kubo, H. M. Grey, and S. Colon, manuscript in preparation.

Table 1. Absorption of the biological activity of AEF on *in vitro* responses of DBA/2 B lymphocytes with immunoadsorbents prepared from rabbit anti-$\beta_2 m$ antisera or normal rabbit serum*

Culture	Cultured DBA/2 cells	AEF (1:10)	Immunoadsorbent for AEF absorption‡	Anti-SRBC PFC response‡
I	Untreated spleen	—	—	1200
II	Anti-θ serum-treated spleen	—	—	0
III		+	None	1140
IV		+	Normal rabbit serum	1530
V		+	Anti-mouse $\beta_2 m$	180
VI		+	Anti-human $\beta_2 m$	170
VII		+	Anti-human $\beta_2 m$ + human $\beta_2 m$	940

* Untreated or anti-θ serum-treated spleen cells from unprimed DBA/2 mice were cultured either alone or with unabsorbed AEF or AEF absorbed on the immunoadsorbent indicated in the presence or absence of SRBC for 4 days.
† AEF was incubated for 1 hr at room temperature with immunoadsorbents prepared from the antisera indicated.
‡ IgM PFC per 10^7 cultured cells; background PFC in unstimulated control cultures have been subtracted from the PFC values obtained in the corresponding SRBC-stimulated cultures. Background PFC values were not altered by incorporation of AEF into the cultures.

acting anti-human $\beta_2 m$ serum are those responsible for its absorptive capacity for AEF.

Failure to absorb AEF activity with anti-immunoglobulin antiserum

It has been amply demonstrated that extensive homologies in amino-acid sequences exist between $\beta_2 m$ and certain constant region domains of immunoglobulin heavy chains; despite this, anti-Ig antisera fail to react with $\beta_2 m$ (reviewed in refs. 19 and 20). In view of the absorptive capacity of anti-$\beta_2 m$ antisera for AEF observed in the preceding experiments, it seemed prudent to ascertain whether or not such reactivity may be due to the presence of Ig antigenic determinants in AEF. As shown in Table 2, immunoadsorbents prepared from either normal rabbit serum or polyvalent rabbit anti-mouse Ig serum failed to absorb any biologically active AEF as tested in the assay system of reconstitution of anti-θ serum-depleted spleen cell responses to SRBC.

DISCUSSION

The experiments presented here demonstrate the capacity of anti-$\beta_2 m$ antisera to react with determinants present on the biologically active moiety of allogeneic effect factor (AEF). This conclusion derives from the findings that antisera directed against a mouse $\beta_2 m$-like protein and cross-reactive anti-human $\beta_2 m$ antisera absorb the biological activity of AEF (Fig. 1, Table 1). The absorption appears to be specific since the absorptive capacity of cross-reactive anti-human $\beta_2 m$ can be inhibited by purified human $\beta_2 m$ (Table 1). Artifacts possibly due to the presence of fetal calf serum in the AEF preparations have been eliminated since the same results are obtained with AEF obtained in serum-free conditions. Moreover, the possibility that the absorption of AEF by anti-$\beta_2 m$ antisera reflects reactivity or cross-reactivity of such antisera with Ig or Ig-like determinants on AEF has been ruled out by the failure of polyvalent anti-Ig antisera to manifest any absorptive capacity for AEF. Taken collectively, therefore, these results strongly indicate that $\beta_2 m$ or an antigenically related entity is associated with the biologically active component of AEF.

In recent years, studies on the structure of histocompatibility antigens have indicated that they are composed of at least two molecules linked noncovalently, one of which is a gene product of the MHC and the other $\beta_2 m$ (20, 25–29). Current structural analyses of AEF being performed in our laboratory have strongly indicated the similarities between AEF and histocompatibility molecules in that dissociative chromatography of purified AEF results in two components differing in molecular size (about 40,000 and 12,000 daltons); the two subfractions have been shown to exert syner-

Table 2. Failure to absorb AEF activity with polyvalent rabbit anti-mouse immunoglobulin antiserum*

Culture	Cultured DBA/2 cells	AEF (1:10)	Immunoadsorbent for AEF absorption†	Anti-SRBC antibody response‡	
				IgM-PFC	IgG-PFC
I	Untreated spleen	—	—	420	450
II	Anti-θ serum-treated spleen	—	—	53	27
III		+	None	890	753
IV		+	Normal rabbit serum	973	807
V		+	Rabbit anti-mouse Ig	927	920

* Untreated or anti-θ serum-treated spleen cells from unprimed DBA/2 mice were cultured either alone or with unabsorbed AEF or AEF absorbed on the immunoadsorbent indicated in the presence or absence of SRBC for 4 days.
† AEF was incubated for 1 hr at room temperature with immunoadsorbents prepared from the antisera indicated.
‡ PFC per 10^7 cultured cells; background PFC in unstimulated control cultures have been subtracted from the PFC values obtained in the corresponding SRBC-stimulated cultures. Background PFC values were not altered by incorporation of AEF into the cultures.

gistic biological activity on B cell responses to antigen *in vitro* (12). The findings presented herein on the absorptive capacity of anti-β_2m for AEF support the probability that β_2m is a structural component of the biologically active entity of AEF. Although they are suggestive, these observations do not indicate on which of the two molecular components the β_2m determinants exist and further analysis is necessary before such conclusions are reached.

In a previous report from our laboratory, we demonstrated the presence of Ia determinants on AEF as evidenced by the specific absorption of AEF activity by anti-Ia antisera (11). The present demonstration of β_2m-like determinants on AEF raises some contradictions which must be addressed here. Briefly, Ia antigens which are most quantitatively evident on B lymphocytes have not been reported to be associated with β_2m or β_2m-like molecules (30, 31). These findings suggest that β_2m is not associated with Ia molecules on the cell surface, although it is clearly associated with other MHC gene products (20, 25–29). On the other hand, our findings suggest a functional association between the Ia and β_2m molecules. There are several possible explanations for this apparent contradiction:

(*1*) Since the Ia molecules are in quantitative excess on B cells as compared to T cells, it is possible that the chemical characterization studies performed thus far have been largely reflecting properties of B cell Ia which may, indeed, not be associated with β_2m.

(*2*) The Ia determinants on AEF presumably are T cell products which may be either identical to or slightly or substantially different in structure and composition than B cell Ia. Even if identical, there may be an association of T cell Ia molecules with β_2m which may not be the case for B cell Ia. Discrimination of these possibilities will be made when isolation and chemical characterization of T cell Ia is performed.

(*3*) Finally, one must be cognizant of the fact that AEF is obtained under remarkably different conditions—i.e., shedding and/or secretion by living cells—than those used for isolation of Ia antigens by immunochemical means. It is conceivable, therefore, that the association of a β_2m chain on B cell Ia has escaped attention as a result of the preparative methods employed and that appropriate modification of such procedures will reveal this association in the future.

The association of β_2m with histocompatibility antigens and the remarkable sequence homologies of the molecule to constant region domains of Ig has been shown in several species (19, 20, 25–29). Hitherto, no apparent biological function could be assigned to this small molecule. However, recent observations have demonstrated that antisera against β_2m may have either enhancing or suppressive effects on certain lymphocyte functions, indicating the possible involvement of β_2m in various immune processes (32–35). Recently, Schimpl *et al.* (36) reported that incorporation of anti-human β_2m antiserum into cultures containing a comparable T cell factor appeared to block the capacity of their factor to stimulate antibody production. Such observations and the results of the present studies indicating an association of β_2m with the biological activity of AEF strongly suggest that β_2m is involved in cell–cell interactions and the consequences of such interactions on lymphocyte triggering and differentiation.

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