Brief Definitive Report

PRESENCE OF INTERCHAIN DISULFIDE BONDS BETWEEN TWO GENE PRODUCTS THAT COMPOSE THE SECRETED FORM OF AN ANTIGEN-SPECIFIC SUPPRESSOR FACTOR*

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An antigen-specific suppression of antibody response has been known to be mediated by the factor (TsF) derived from suppressor T cells in a variety of experimental systems. An antigen-specific TsF has also been found to bear the products of genes in the I-J subregion of the H-2 complex and the antigen-binding moiety having no known constant-region determinants of the immunoglobulins (1–7). Although the nature of the antigen-binding structure of TsF is still largely unknown, recent reports from various laboratories (8–11) have indicated that TsF carries the determinants analogous to the variable region of the immunoglobulin heavy chain. It is, therefore, conceivable that at least two gene products are involved in the structural entity of an antigen-specific TsF: each product coded for by genes in one locus or the other.

Despite the information mentioned above, the structure of an antigen-specific TsF is still controversial. Our recent studies using the extracted TsF from a T cell hybrid specific for keyhole limpet hemocyanin (KLH) have strongly suggested that the TsF is composed of the two distinct polypeptide chains, i.e., the antigen-binding and the I-J-encoded chains in noncovalent association (7). On the other hand, Kontiainen et al. (5) have indicated that these two determinants on the secreted TsF specific for KLH are present on the same molecule. It is therefore of importance to determine the structural entity of an antigen-specific TsF.

In this communication, we have further examined the structural and functional organization of the secreted form of the KLH-specific TsF from the hybridoma 34S-704. We hereby demonstrate that the secreted TsF is composed of two distinct polypeptide chains, i.e., the one having the antigen-binding moiety and the other having the I-J subregion gene product, in association with disulfide bonds. Furthermore, we show that the two dissociated polypeptide chains obtained after the reduction of the TsF with dithiothreitol (DTT) can successfully reassociate and reconstitute the TsF activity if the reduced TsF is not alkylated, and that the association of these two chains is essential for the expression of the TsF activity.

Materials and Methods

Animals. C57BL/6 mice were purchased from the Shizuoka Experimental Animal Laboratory Co., Ltd., Hamamatsu, Japan.

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Antigens. KLH was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Dinitrophenylated KLH (DNP-KLH) was prepared by coupling with 2,4-dinitrobenzensulfonic acid under an alkaline condition by the method of Eisen et al. (12). Bordetella pertussis vaccine (BPV) was purchased from the Chiba Serum Institute, Chiba, Japan.

Immunoadsorbent Columns. 10 mg of KLH or 1 ml of gammaglobulin fraction of B10.A(5R) anti-B10.A(3R) antiserum (anti-I-J^b) was coupled with 1 ml of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method of Axen et al. (13).

Preparation of KLH-specific TsF (KLH-TsF). The KLH-TsF obtained from the ascitic fluid of the hybridoma-bearing mice was used. The T cell hybridoma 34S-704 was made by fusion of the AKR thymoma BW5147 and KLH-specific suppressor T cells from KLH-primed spleen cells of C57BL/6 mice enriched with KLH-coated Petri dishes as described previously (14). The properties of the TsF derived from hybridoma 34S-704 have been described elsewhere (6). 1×10^8 hybrid cells (34S-704) were intraperitoneally inoculated into (C57BL/6 × AKR)F_1 mice. 10 d later, ascitic fluid was collected and 0.5 mM phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, Mo.) was added. They were stored in liquid nitrogen (−160°C).

Purification of the KLH-TsF. The ascitic fluid from the hybridoma 34S-704-bearing mice was applied to an immunoadsorbent column composed of KLH. It was incubated for 1 h at 4°C, and the column was extensively washed with 0.01 M sodium phosphate-buffered saline (PBS), pH 7.2. The active materials were then eluted from the column with 0.175 M glycine-HCl buffer, pH 3.2. The eluted materials were dialysed with 0.01 M PBS, pH 7.2, and were concentrated by vacuum pressure.

Reduction and Alkylation of the KLH-TsF. The purified KLH-TsF was reduced with 5 mM DTT at room temperature for 45 min. The reduced materials were subsequently alkylated with 10 mM iodoacetamide at room temperature for 20 min. The reaction was carried out in 0.15 M Tris-HCl buffer, pH 8.0, containing 2 mM EDTA under the condition of saturated N_2. The materials were extensively dialysed overnight against 0.01 M PBS, pH 7.2 and concentrated by vacuum pressure. In some experiments, the purified TsF was treated with only 5 mM DTT or 10 mM iodoacetamide under the above conditions.

Assay for the TsF Activity. The TsF activity was assayed in the in vitro secondary antibody response against DNP-KLH as described in the previous paper (6). Briefly, 4×10^6 spleen cells from C57BL/6 mice primed with 100 μg DNP-KLH plus BPV 4 wk earlier were cultured by the Mishell-Dutton system in the presence of 0.1 μg/ml of DNP-KLH at 37°C in 5% CO_2. The TsF was added to the culture on day 0. 5 d later, the numbers of anti-DNP IgG plaque-forming cells (PFC) were assayed by using DNP-coupled sheep erythrocytes.

Statistics. For in vitro assays, results were analyzed with a two-tailed Student's *t* test.

Results and Discussion

Our previous studies (6) have shown that the KLH-TsF in the extracted or the secreted materials derived from the T cell hybridoma 34S-704 carries the antigen-binding moiety and the I-J-encoded determinants. Furthermore, by using the extracted TsF, these two distinct determinants have been found to be on the different polypeptide chains in noncovalent association. On the other hand, the secreted TsF has different features from the extracted TsF, possessing the two characteristic determinants on the same molecule (7). These data have therefore indicated that the antigen-binding and the I-J-encoded polypeptide chains are noncovalently linked in cytoplasm or on the cell surface, and that they are secreted after their covalent association.

To test this possibility, the secreted TsF was examined to determine the presence of the interchain disulfide bonds and to cleave into the two polypeptide chains. 800 μl of the ascites from the hybridoma 34S-704-bearing mice was applied to a KLH column. The active KLH-TsF was then eluted with 0.175 M glycine-HCl buffer, pH 3.2. 800 μl of the purified TsF was reduced with 5 mM DTT and alkylated with 10

TABLE I

Reduction and Alkylation of the Secreted KLH-TsF Derived from a T Cell Hybridoma

KLH-TsF treated with	Anti-DNP IgG PFC/culture*	Significance of difference‡
—	2,394 ± 301	
Untreated	798 ± 120	$P < 0.01$
5 mM DTT and 10 mM iodoacetamide	2,100 ± 180	$P > 0.3$
5 mM DTT	725 ± 139	$P < 0.01$
10 mM iodoacetamide	651 ± 138	$P < 0.01$

The secreted KLH-TsF purified with the KLH column was reduced and/or alkylated as indicated in Materials and Methods. The materials were then tested for their activity in the in vitro secondary antibody response.

* Arithmetic mean numbers of anti-DNP IgG PFC of four cultures ± SD.

‡ Student's *t* tests, when compared with the control group.

mM iodoacetamide. In other experiments, the purified TsF was treated only with 5 mM DTT or with 10 mM iodoacetamide under the same conditions as above. The materials thus obtained were extensively dialysed and tested for their activity in the culture of spleen cells primed with DNP-KLH plus BPV. As shown in Table I, the suppressor activity of the purified TsF was completely abrogated by the reduction and alkylation. In contrast, the activity was still preserved by the treatment of the TsF with only one of DTT or iodoacetamide. Although there is no direct evidence that the treatment of the TsF with 5 mM DTT does not cleave the intrachain disulfide bonds, the same treatment has been demonstrated not to affect the intrachain disulfide bonds of immunoglobulins (15). Therefore, these results strongly suggest that there are interchain disulfide bonds between the antigen-binding and I-J-encoded polypeptide chains of the secreted TsF, and that the cleaved chains successfully reassociate and reconstitute the active form of the TsF.

Because our previous studies (7) using the extracted TsF have clearly indicated that the association of the antigen-binding and the I-J chains is necessary for the expression of the TsF activity, we therefore tried to test whether the suppressor activity obtained by the reduced TsF is due to the reassociation of the cleaved two chains of the secreted TsF.

The secreted KLH-TsF reduced with 5 mM DTT was applied to the KLH column equilibrated with Tris-HCl buffer, pH 8.0, containing 0.5 mM DTT, in order to separate the antigen-binding polypeptide chains from the I-J products. The materials bound to the KLH column were eluted with 0.175 M glycine-HCl buffer, pH 3.2. The effluent, the eluate, and the mixture of the half volume of these two were tested for their activity. As shown in Fig. 1, the suppressor activity was still preserved in the reduced materials of the TsF. However, when they were fractionated by the KLH column, no significant activity was observed in the effluent or the eluate. On the other hand, the mixture of the half volume of the effluent and the eluate reconstituted strong suppressor activity. This indicates that the combination of the materials in the effluent and those in the eluate composes the active TsF.

Further, to investigate whether the two distinct materials in the effluent and the eluate of the reduced TsF from the KLH column (1st column; designated as the effluent KLH and the eluate KLH) are in fact the antigen-binding and I-J-encoded polypeptide chains, the half volume of the dialysed effluent or eluate from the KLH

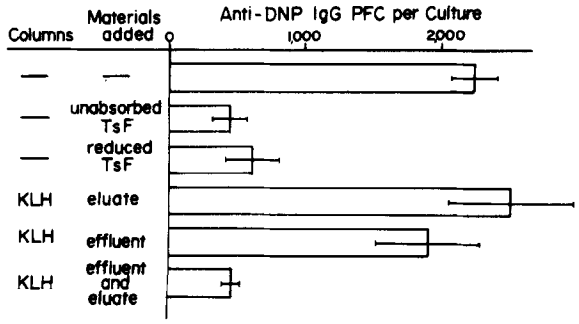

FIG. 1. Successful reconstitution of the TsF activity in the mixture of the effluent and the acid eluate from the KLH column having absorbed the reduced TsF. The secreted TsF reduced with 5 mM DTT was fractionated by the KLH column in the presence of 0.5 mM DTT. The effluent, the eluate, and the mixture of the half volume of the two were tested for their activity. The bars indicate the mean PFC numbers ± SD of four cultures. Student's t tests, unabsorbed TsF, $P < 0.01$; reduced TsF, $P < 0.01$; eluate, $P > 0.5$; effluent, $P > 0.2$; eluate + effluent, $P < 0.001$.

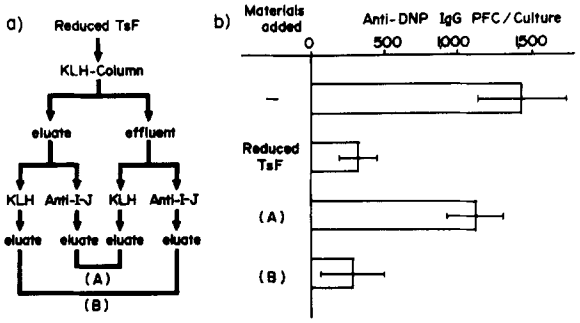

FIG. 2. Reconstitution of the TsF activity with the combination of the antigen-binding and I-J-encoded chains. The half volume of the effluent or the eluate of the reduced TsF from the KLH column was successively absorbed with the KLH and anti-I-J^b columns. The acid eluate from the column were mixed and made two groups, A and B, as indicated in panel a. The activity of group A or B was tested and demonstrated in panel b. The bars indicate the mean PFC numbers ± SD of four cultures. Student's t tests. Reduced TsF, $P < 0.01$; group A, $P > 0.3$; group B, $P < 0.05$.

column, each of which had no activity, was successively absorbed with the KLH and anti-I-J^b columns. The acid eluates from the second columns were mixed and made into two different groups (A and B) as indicated in Fig. 2a. Group A consists of the mixture of the eluate from the second KLH column, having absorbed the effluent KLH, and that from the anti-I-J column having absorbed the eluate KLH. Similarly, group B is composed of the mixture of the eluate from the anti-I-J column, having absorbed the effluent KLH, and that from the second KLH column having absorbed the eluate KLH. As shown in Fig. 2b, group B gave a strong suppressor activity, whereas no detectable effect was observed by group A. From the results, it is clear that the antigen-binding and I-J-encoded polypeptide chains of the secreted TsF are cleaved by the DTT treatment, and it is most likely that they are easy to reassociate to compose the active TsF, although the experiments do not exclude the possibility that the two distinct chains without direct linkage would be active on the target cells.

The polypeptide chain with antigen-binding capacity per se could directly bind to the antigen but not exert the TsF activity, so that it acts as the element for the antigen-specificity of the TsF. Concerning the role of the I-J product on TsF, it should

act as a functional molecule to convey suppressor activity. However, this does not imply that the I-J products themselves could exert their suppressor function, because the effluent of the reduced TsF from the KLH column, which should contain I-J products, did not show any suppressor activity (see Fig. 1). Therefore, it can be concluded that the KLH-TsF is composed of the two distinct gene products of the KLH-binding and I-J-encoded polypeptide chains, and that the association of these two chains is essential for the expression of the KLH-TsF function. In this sense, the covalent association of these two chains with disulfide bonds may keep the TsF more stable even after they were secreted.

In any event, the KLH-TsF reported in this paper resembles other antigen-specific T cell factors with suppressor function reported by Theze et al. (2), and Kontiainen et al. (5), and with helper function as described by Taussig et al. (16), Isac et al. (17), and Tokuhisa et al. (18). In particular, the antigen-specific helper T cell factors have also demonstrated that they have the antigen-binding moiety and the I-A-encoded determinants. Therefore, it appears that the I-region gene products (i.e., I-A or I-J products) detected on the antigen-specific helper and suppressor factors in association with the antigen-binding moiety directly reflect the function of the T cell factors.

Summary

The secreted form of the suppressor T cell factor specific for keyhole limpet hemocyanin derived from the hybridoma 34S-704 was found to consist of the two distinct polypeptide chains, i.e., the antigen-binding and the I-J-encoded chains. They were linked in covalent association with disulfide bonds. The two chains were cleaved by the reduction with dithiothreitol and were easy to reconstitute the active form of TsF. The association of the two distinct chains was suggested to be essential for the expression of the TsF activity.

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