TWO TYPES OF ANTIGEN-BINDING T-SUPPRESSORS FORMED DURING THE IMMUNE RESPONSE CHARACTERIZED BY MEANS OF T-T HYBRIDOMAS AND THEIR EXTRACTS

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Recent investigations have shown that T hybridomas obtained by fusion of different populations of functionally active T cells and mouse thymomas or myelomas preserve the function of an immune T cell, carry its differential antigens (AG), and synthesize the corresponding factors [3, 9, 10, 15]. Consequently, T hybridomas can be used to study the functional properties of different subpopulations of T cells [9, 15].

It was shown previously that reactions of delayed-type hypersensitivity (DTH), induced by intravenous injection of 10^5 syngeneic spleen cells modified by 2,4,6-trinitrophenyl sulfonic acid or azobenzene arsonate (TNP- and ABA-SC, respectively) are accompanied by accumulation of antigen-binding T suppressors (Ts), specifically inhibiting the afferent component of compact sensitivity to 2,4,6-trinitrochlorobenzene (TNCB) or DTH induced by subcutaneous injection of $3 \cdot 10^7$ hapten-modified spleen cells, in syngeneic recipients [5, 6].

The aim of this present investigation was to obtain antigen-binding hybridomas of T suppressors formed during the immune response and to investigate their properties and the properties of their extracts.

EXPERIMENTAL METHOD

BALB/c mice weighing 18-20 g, obtained from the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR and (AKR × BALB/c)F_1 mice, bred at the vivarium of the Institute of Immunology, Ministry of Health of the USSR, were used. An enriched population of splenic T cells from mice immunized intravenously, 6 days previously, with 10^5 TNP-SC, and capable of adsorption on plastic dishes coated with TSP-bovine serum albumin (TNP-BSA) [6], and a subclone of thymoma BW5147.3.13 (BW3.13), not possessing nonspecific suppressor activity and resistant to culture in the presence of 10 μg/ml of 6-thioguanine [4, 7] were used for hybridization. Hybridization was carried out by the method described previously [1]. The fused cells (10^4 per well) were cultured in 96-well plates in DMEM medium containing 20% embryonic calf serum (from Flow Laboratories, England), 60 U/ml of gentamicin, 10^{-4} M hypoxanthine, $4 \cdot 10^{-7}$ M aminopterin, and $1.6 \cdot 10^{-5}$ M thymidine (from Sigma, USA). Peritoneal macrophages (10^4 per well) were used as the nurse layer.

Hybridomas were selected for antigen-binding capacity in the rosette formation test with sheep's red blood cells (SRBC), conjugated with the aid of $CrCl_3 \cdot 6H_2O$ with TNP-BSA [8]. Either ABA-BSA-SRBC or SRBC were used as the control. Lyt- and Thy-antigens were determined by the indirect immunoflorescence test, with recording in a fluorescence microscope [1] or on an Ortho 50-H flow cytometer (USA) [2]. Monoclonal rat antibodies (McAB) against Thy 1.2 antigens [14] were generously provided by R. G. Vasilov. Suppressor activity of the hybrid cells was investigated by transfer into BALB/c mice, which were sensitized subcutaneously either with a 5% solution of TNCB in 100 μl of alcohol or at two points in the spinal region with $3 \cdot 10^7$ TNP- or ABA-SC [6]. The development of DTH was tested on the 6th day after sensitization by the

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TABLE 1. Phenotypic Characteristics and Suppressor Activity of Antigen-Binding T-T Hybridomas

Cell cultures	RFC, %			Cells carrying AG, %				Suppression of DTH, %	
	TNB	ABA	SRBC	Thy 1.1	Thy 1.2	Lyt 1	Lyt 2	by cells	by culture fluid
BW3.13	6	3	3	>90	0	0	0	0	0
2.A1	30	N.t.	2	>90	0	0	0	0	0
2.B6	44	5	5	>90	0	0	30	N. t.	0
FL2 B8	55	3	2	>90	0	0	30	68	0
IC7	67	N. t.	N. t.	>90	20	20	0	0	0
ID4	50	N. t.	N. t.	>90	>90	0	>90	0	Stimulation
Fi2G4	53	2	3	>90	>90	>90	30	43	0

Legend. N.t.) Not tested.

skin test method [6]. For this purpose, during sensitization with TNCB 25 µl of a 1% solution of TNCB in olive oil was applied to the skin of the ear. During sensitization with TNP-SC 130 µg of TNPS in 40 µl of Hanks' solution, pH 7.4, was injected intradermally into the hind foot pad. Testing with ABA was carried out also in the foot pad, and in this case 50 µg of ABA in 15 µl of Hanks' solution, pH 8.2, was used. The reaction was read after 24 h by means of an MK 0-25 engineer's micrometer. The difference in thickness of the ears and feet characterized the degree of edema and the intensity of the reaction. The control in these experiments consisted of sensitized animals (control$^+$) and intact mice on which skin tests were carried out (control$^-$). The percentage suppression of DTH was calculated as follows:

$$1 - \frac{\text{degree of edema in experimental group} - \text{degree of edema in control}^-}{\text{degree of edema in control}^+ - \text{degree of edema in control}^-} \times 100\%.$$

The suppressor activity of the culture fluid of 48-h cultures of hybridomas or extracts obtained by freezing 5 times (to −60°C) and thawing (37°C), followed by centrifugation at 15,000g for 40 min, was estimated in the same way. The culture fluid and extracts were injected intravenously in doses equivalent to $5 \cdot 10^6$ cells. Extracts for testing suppressor activity in the phase of expression of DTH were injected before the skin tests were done.

To multiply the hybridoma cells *in vitro*, $15 \cdot 10^6$ hybridoma cells, enriched on plastic dishes, coated with antigen [6], were injected intraperitoneally into (AKR × BALB/c)F_1 mice. An injection of 0.5 ml of Pristan (from Sigma) was given to the mice 7-14 days before injection of the cells.

EXPERIMENTAL RESULTS

On the 4th week of culture, 13 of the 18 growing hybridomas were tested for ability to form rosettes with TNP-SRBC. The results (Table 1) show that 6% were able to bind antigens and to form rosettes with TNP-SRBC, but not with ABA-BSA-SRBC. Some antigen-binding hybridomas expressed Lyt and Thy 1.2 antigens of the immune partner, and two of them (FL2B8 and FI2G4) suppressed the phase of induction of contact sensitivity to TNCB.

The fact will be noted that not all cells of antigen-binding (RFC$^+$) hybridoma cultures bound AG and not all cells of a culture expressing a certain differential AG did in fact express this AG. This is in agreement with previous findings, showing that expression of antigen-binding receptors and of differential AG on cells of one culture may vary considerably depending on the cell cycle and the conditions of culture [16].

It must be pointed out that the suppressor hybridomas obtained were unstable with respect to expression of antigen-binding receptors and expression of differential AG. On retesting (2 weeks later) ability to bind AG and determination of Lyt antigens, it was discovered that the percentage of RFC in hybridomas FL2B8 and FI2G4 was reduced from 50 and 60% to 17 and 20%, respectively, and that both hybridomas had lost their Lyt 2 antigen.

Testing the suppressor activity of cells from cultures of hybridomas FL2B8 and FI2G4, which preserved about 20% of their antigen-binding cells, at the 6th week of culture showed that it was maintained at a high level (Fig. 1). Suppression of the phase of DTH induction was specific, and not only hybridoma cells, but also extracts obtained from these cells possessed

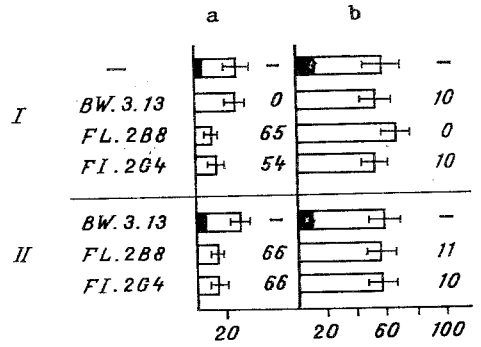

Fig. 1

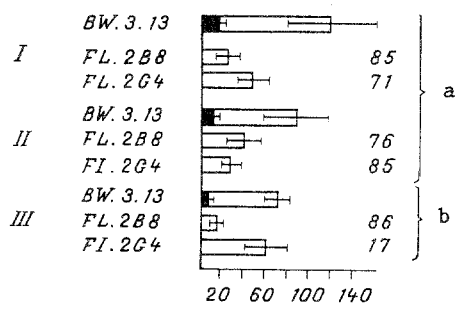

Fig. 2

Fig. 1. Effect of injection of $5 \cdot 10^6$ suppressor hybridoma cells (I) and equivalent doses of extracts (II) on intensity of DHT reactions induced by TNCB or ABA-SC. Horizontally — degree of edema (in $mm \cdot 10^{-2}$). Numbers on right of columns indicate percentage suppression of DTH. Positive control — sensitized animals. Black part of column denotes degree of edema in negative control. Hybridoma cells and extracts injected intravenously before sensitization. Mice tested with corresponding antigen 6 days later. a) testing with TNCB, b) with ABA.

Fig. 2. Effect of injection of extracts of suppressor hybridomas in different phases of DTH (a — induction; b — expression) on intensity of skin reactions in mice sensitized by $3 \cdot 10^7$ TNP-SC. I, II, III) injection of extracts: I) Exp. No. 1, II) Exp. No. 2, III) Expts. Nos. 3 and 4. Extracts in doses equivalent to $5 \cdot 10^{-6}$ cells were injected intravenously either before sensitization of the mice (induction phase) or before testing with 2,4,6-trinitrophenylsulfonic acid (expression phase). The level of reactions in animals injected with the analogous dose of extract BW3.13 was taken as the positive control. Remainder of legend as to Fig. 1.

suppressor activity. The suppressor hybridomas were evidently incapable of secreting suppressor factors *in vitro*, for the culture fluid of both hybridomas was without suppressor activity (Table 1).

Considering data showing that periodic enrichment of antigen-binding cells of the hybridoma cultures is an essential condition for the preservation of suppressor activity [9], later, before multiplication of the hybridomas *in vivo*, in order to obtain extracts from them, this method was used. Data on suppression of the phase of induction and expression of DTH by extracts of cells multiplied *in vivo* are given in Fig. 2. It will be clear from Fig. 2 that the hybridomas thus obtained differed in their functional properties. Extract Lyt $1^-,2^+$ of hybridoma FL2B8 suppressed both the phase of induction and the phase of expression of DTH, whereas extract Lyt $1^+,2^+$ of hybridoma FI2G4 inhibited only the induction phase of DTH.

Antigen-binding hybridomas Lyt $1^-,2^+$, extracts of which suppressed the expression phase of DTH, were described previously [9, 15]. They were hybrids of Ts-effectors (TsE), formed in lymph nodes in response to induction of DTH by subcutaneous injection of AG. The results of the present experiments show that on induction of DTH by intravenous injection of AG, cells with a similar Lyt phenotype and with similar functional properties also were formed. In this case the site of formation of this type of Ts evidently depends on the method of induction of DTH, and during intravenous immunization functional equivalents of TsE are formed in the spleen.

Suppression of the immune response by Lyt $1^+,2^+$ suppressors has been described during induction of antibody formation to SRBC *in vitro* [11] and during immunization of mice with lactate dehydrogenase B [12]. In the first model Lyt $1^+,2^+$ Ts are acceptors of the suppressor factor produced by Lyt $1^+,2^-$ Ts inducers. In the second model Lyt $1^+,2^+$ Ts effectors suppressing proliferation of Lyt 1^+ cells are described. In both cases the suppressor effect of Lyt $1^+,2^+$ suppressor cells and of the factors produced by them, as well as the suppressor activity of an extract of hybridoma FI2G4, were manifested in the phase of induction of the immune response.

On injection of tolerogenic doses of AG suppression of the induction phase of DTH was due to Lyt $1^+,2^-$ Ts inducers [9]. Our experiments showed that suppression of the induction phase of DTH may also be due to Lyt $1^+,2^+$ cells. However, it is not known whether these cells are

an intermediate stage of differentiation of Ts inducers, detected in the presence of tolerance, or whether they are the functional equivalents of Lyt $1^+,2^+$ Ts effectors [12], suppressing proliferation of Lyt 1^+ cells.

The data given in this paper are thus evidence that in the course of the immune response, during induction of DTH by intravenous injection of syngeneic spleen cells, modified by a hapten, parallel with the formation of effectors of DTH [5], the formation of two types of antigen-binding Ts, differing from one another in their Lyt phenotype and functional properties, takes place in the spleen of the experimental animals.

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LIPID PEROXIDATION IN THE RAT LIVER DURING STIMULATION OF MACROPHAGES

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Most tumoricidal and bactericidal effects of macrophages are known to be connected with the ability of these cells to produce active forms of oxygen (the superoxide radical, hydrogen peroxide, the hydroxyl radical, and singlet oxygen) [14]. Phagocytosis and various surface-active factors, including adjuvants, chemotaxic peptides, lectins, endotoxins, and so on, lead to characteristic metabolic changes in macrophages, accompanied by a "burst" of oxygen consumption and by an increase in the production of reactive metabolites of oxygen [6]. Considering the high ability of activated forms of oxygen to initiate free-radical lipid peroxidation (LPO) reactions in membranes, and also their free liberation into the extracellular medium [8], it is logical to suggest that induced macrophages participate in the realization of the pro-oxidant effect *in vivo*. Nevertheless, the protective action of zymosan, a stimulator of the mononuclear phagocyte system (MPS), under conditions of lesions of radiation or free-radical nature is difficult to explain from this point of view [1]. In the present investigation accumulation of one end product of LPO (malonic dialdehyde, MDA) in the animal

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