Immunology 1986 **57** 545–552

Cellular requirements for the suppression of leucocyte adherence inhibition reactions by serum factors from tumour-bearing mice

V. K. KUCHROO & W. J. HALLIDAY *Department of Microbiology, University of Queensland, St Lucia, Brisbane, Australia*

Accepted for publication 27 November 1985

SUMMARY

Tumour-bearing mice produce circulating serum factors that block cell-mediated immunological reactions *in vitro*. The mechanism by which these specific suppressor factors (SF) block leucocyte adherence inhibition (LAI) was studied. It had previously been shown that the antigen-reactive effector cells in the LAI assay are Ly-1$^+$ T cells. We have now found that Ly-2$^+$, I-J$^+$ cells are required in the reactive cell population to observe the blocking action of SF from serum. Tumour-bearer spleen cells (containing Ly-2$^+$, I-J$^+$ lymphocytes) reacted only with the specific tumour-related serum factor (SF1) and relevant tumour antigen, to produce a non-specific suppressor factor (SF2). Specificity studies were conducted with contact-sensitized mice: hapten-specific spleen cells reacted only with hapten-related SF1 and the relevant hapten, to produce a similar SF2. SF2 differed from SF1 in suppressing allogeneic as well as syngeneic cells, in suppressing populations depleted of Ly-2$^+$, I-J$^+$ cells, and in being unaffected by absorption with immobilized anti-I-J antibody. Gel-filtration of SF2 revealed two forms of differing MW ($>$ 190,000 and 20,000–50,000).

INTRODUCTION

Immunosuppression is a well-recognized consequence of cancer, and the suppression of immunoreactivity is one of the ways in which neoplastic cells are thought to escape the host's defences. The detection, characterization and mode of action of suppressor cells and suppressor factors (SF) have been intensively studied, especially in experimental cancer of animals and in other model systems. Much of this research has been related to the suppression of cell-mediated immunity (CMI). The 'blocking factors' in cancer sera were the first examples of tumour-related specific SF detected by *in vitro* techniques (Hellström & Hellström, 1969); other recent work has concentrated on the suppression of antibody and CMI to haptens, the SF being assayed in cell extracts of culture fluids, usually by *in vivo* methods (Greene, Bach & Benacerraf, 1979; Zembala, Asherson & Colizzi, 1982a; Zembala *et al.*, 1982b; Ptak, Gershon & Flood, 1983; Dorf & Benacerraf, 1984).

Previous studies from this laboratory have employed leucocyte adherence inhibition (LAI) to detect antigen-specific SF in sera from cancer patients and tumour-bearing animals (Halliday & Miller, 1972; Halliday *et al.*, 1980; Koppi, Halliday & McKenzie, 1981), as well as from mice rendered tolerant to contact-sensitizing haptens (Noonan & Halliday, 1978; Halliday & Noonan, 1978). LAI is an *in vitro* assay that depends on the rapid liberation of a lymphokine from antigen-stimulated Ly-1$^+$ T lymphocytes, and the subsequent inhibition by the lymphokine of the glass-adherence of indicator leucocytes (Powell, Sloss & Smith, 1978; Koppi & Halliday, 1982). SF from sera or culture fluids are detected by their ability to counteract LAI. We have shown that the SF studied in mice are specific for tumour antigens or sensitizing haptens, exist in two forms of MW 30,000–50,000 and $>$ 100,000, are produced by Ly-2$^+$, I-J$^+$ T lymphocytes in culture, and the smaller form from serum has antigen-binding and I-J sites on separate polypeptide chains (Halliday, Koppi & McKenzie, 1982; Koppi & Halliday, 1983; Koppi-Reynolds & Halliday, 1984).

Although leucocytes from tumour-bearing animals had previously been shown to retain their LAI reactivity after treatment with anti-Ly-2 antibody and complement (Koppi & Halliday, 1982), such treated cells had never been used to test for suppression. Following the report of Zembala *et al.* (1982b), describing production of a secondary SF by Ly-2$^+$ lymphocytes from contact-sensitized mice, we found that analogous cells from tumour-bearing animals also played a special role in the manifestation of suppression. Several of the characteristics of the system were different from those previously described.

Abbreviations: CMI, cell-mediated immunity; FCS, fetal calf serum; LAI, leucocyte adherence inhibition; PC, peritoneal cells; PSA, picrylsulphonic acid; SC, spleen cells; SF, suppressor factor(s); SF1, primary (serum) suppressor factor; SF2, secondary suppressor factor; TNCB, 1-chloro-2,4,6-tri-nitrobenzene; TNP-BSA, trinitrophenylated bovine serum albumin.

Correspondence: Dr V. K. Kuchroo, Dept. of Microbiology, University of Queensland, St Lucia, Brisbane, Australia.

MATERIALS AND METHODS

Mice

Young adult CBA and BALB/c mice were provided by the Central Animal Breeding House, University of Queensland.

Tumours

A fibrosarcoma induced in CBA mice by 3-methylcholanthrene and designated MCA2 was used throughout the present study. The tumour was maintained by transplantation in syngeneic mice and these mice were used as a source of peritoneal cells (PC) or spleen cells (SC) 10–16 days after transplantation. A BALB/c transitional cell bladder carcinoma (1660) was obtained from Drs I. and K. E. Hellström (Oncogen, Seattle, WA) and was serially transplanted in BALB/c mice in our laboratory.

Sensitization and tolerization

Mice were contact-sensitized to 1-chloro-2,4,6-trinitrobenzene (TNCB, picryl chloride; British Drug Houses, Poole, Dorset, U.K.) by the single application of 4 mg in 0·1 ml of acetone to a skin area of approximately 5 cm^2 on the abdomen. Mice to be made tolerant to TNCB were given a single dose of 5 mg of neutralized picrylsulphonic acid (PSA; Sigma Chemical Co., St Louis, MO) in 0·1 ml of saline, by intraperitoneal injection, followed 7 days later by a sensitizing dose of TNCB as described above. The sensitized and tolerized mice were skin tested 5 days later by painting TNCB on the ears and measuring the ear-swelling after 24 h. These methods have been described previously (Noonan & Halliday, 1978).

Tumour extracts

Fresh or frozen tumour tissue was weighed and chopped into small pieces. The tissue was homogenized in phosphate-buffered saline (PBS) and then centrifuged, after which the supernatant was retained and frozen in small amounts. The detailed methods for preparation of tumour extracts, determining their specificity and the optimal concentration for use in the LAI assay, have been published previously (Koppi & Halliday, 1982).

Peritoneal cells

PC were obtained from peritoneal washings, collected in Hanks' solution (Commonwealth Serum Laboratories, Parkville, Victoria) containing 5 IU heparin/ml. Cells from four to six mice were pooled, washed with Eagle's basal medium (Commonwealth Serum Laboratories) containing 10% fetal calf serum (FCS), and adjusted to 1×10^7 cells/ml.

Spleen cells

Spleens from normal and tumour-bearing mice were removed, teased apart and passed through stainless steel mesh. Erythrocytes were removed by treating the cell suspension with ammonium chloride. The remaining cells were washed twice with Eagle's medium containing 10% FCS and adjusted to the required concentration.

Hapten conjugates

Trinitrophenylated bovine serum albumin (TNP-BSA) was prepared by published methods (Hudson & Hay, 1980). Preliminary studies showed that 6·25 μg/ml (final concentration) of TNP-BSA was optimal for use as an antigen in the LAI assay.

Haptenized SC (TNP cells)

Normal SC from CBA mice were haptenized with PSA and used as a source of antigen for the production of SF2 in certain experiments. SC suspension ($3–5 \times 10^7$/ml) was mixed with an equal volume of 10 mM PSA adjusted to pH 7·4. The mixture was then incubated at room temperature for 30 min with regular mixing. The cell suspensions were then washed with 10 mM glycylglycine in Eagle's medium (pH 8·0) and further washed three times with chilled Eagle's medium, in order to remove any free PSA. These TNP cells were mixed in equal numbers with armed SC for the production of SF2, as described below.

Mouse sera

Normal, tumour-bearing and tolerized mice were bled from the tail, the blood was allowed to clot, and serum was stored in small quantities at −20° until required.

Depletion of lymphocyte subpopulations

PC or SC were depleted of Ly-2^+ and I-J^+ lymphocytes for several of the experiments. Monoclonal anti-Ly-2·1 antibody and anti-I-J^k antiserum and a control anti-Ia^s antiserum were provided by Prof. I. F. C. McKenzie, Department of Medicine, University of Melbourne. Their production and properties have been described previously (Koppi *et al.*, 1981; Koppi & Halliday, 1982). The cells were treated with the above antibodies by incubation in appropriate dilutions (3×10^7 cells in 1 ml of antibody preparation) at 37° for 30 min. The Ly-2·1 monoclonal antibody was used at a dilution of 1:100, whereas anti-I-J^k antiserum was used at a dilution of 1:4. Controls contained no antibody. Each mixture was then centrifuged and the cells were washed in cold medium. The supernatant was discarded and 2·0 ml of guinea-pig complement (diluted 1:5 in medium) were added to the cell pellet. The cells were resuspended and incubated for a further 30 min at 37°, after which they were washed and suspended in medium (1×10^7/ml).

Suppressor factors (SF)

The primary SF in mouse serum (SF1) was obtained from two sources: serum from mice 10–15 days after transplantation with tumour MCA2 (MCA2-bearer serum), and serum from mice rendered tolerant to TNCB (PSA-TNCB tolerant serum).

Secondary SF (SF2) was produced by the arming of sensitized SC with SF1 from either source and subsequent stimulation by appropriate antigen. In general, $3–5 \times 10^6$ SC in 0·1 ml medium were mixed with 0·05 ml of serum containing SF1 (or normal serum) and incubated for 1 hr. The armed SC were washed twice and then stimulated with antigen (tumour extract or TNP cells) during a further 2 hr incubation. After centrifuging, the supernatants were tested for suppression in the LAI assay. Further details are given later in footnotes to tables.

Gel-filtration

A column (90 × 2·5 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with PBS and calibrated with proteins of known MW. Samples to be fractionated (2-ml) were run through the column with PBS at 20 ml/hr and 5-ml fractions were collected. Selected fractions were pooled together and concentrated to 1/50 volume with Minicon B15 concentrators (Amicon Corp., Lexington, MA).

LAI technique

The direct LAI assay was used and has been described in detail

elsewhere (Koppi *et al.*, 1981; Koppi & Halliday, 1982). The technique involves the determination of the glass-adherence of sensitized PC and the inhibition of this adherence by relevant antigen; SF are detected by their ability to prevent LAI when added to a reactive cell–antigen mixture. For the detection of LAI, the mixtures contained sensitized PC (10^6 cells in 0·1 ml of medium), 0·05 ml of relevant antigen at optimal concentration (or control with medium only) and 0·05 ml of normal mouse serum. For the detection of SF in test sera, these were included in mixtures in place of normal serum. For the detection of SF in culture supernatants, these were used in 0·1-ml amounts to suspend the PC, in place of medium. Thus, the serum concentration was always constant. The mixtures were preincubated at 37° for 30 min and then introduced into haemocytometers (four chambers for each different mixture). After a further incubation at 37° for 60 min, the total cells in each of 20 squares (a pattern of five in each chamber) were counted for each mixture. The non-adherent cells were washed away and the remaining adherent cells were recounted in the same squares. All mixtures were coded before counting. The percentage leucocyte adherence was calculated for each mixture (20 values/mixture), and the mean percentage adherence and standard error (SE) determined. A reduction in adherence, consequent upon the addition of antigen to PC in a mixture with normal serum, was interpreted as LAI. If substitution of a test serum or culture supernatant restored the adherence, this was interpreted as suppression of LAI. The statistical significance of these differences was determined by Student's *t*-test.

RESULTS

Properties of the serum SF

As has been demonstrated and reported many times previously, serum SF detected by LAI in several situations is antigen-specific (Halliday & Noonan, 1978; Koppi *et al.*, 1981). In Table 1, this is shown for the SF related to two antigens employed in the present study. Tumour-bearer serum (MCA2-SF1) specifi-

Table 1. Specificity of serum suppressor factors (SF1)

PC donor	Antigen	Serum donor	% adherence (mean ± SE)	Interpretation
MCA 2-bearer	—	Normal	80·7 ± 4·2	
MCA 2-bearer	MCA 2 ext.	Normal	52.4 ± 2·7*	Significant LAI
MCA 2-bearer	MCA 2 ext.	MCA 2-bearer	84·9 ± 2·9	MCA2-SF 1 suppresses LAI
MCA 2-bearer	MCA 2 ext.	PSA-TNCB tolerant†	64·5 ± 3·8*	PSA-SF1 does not suppress LAI
TNCB-sensitized	—	Normal	77·9 ± 2·7	
TNCB-sensitized	TNP-BSA	Normal	63·5 ± 1·8*	Significant LAI
TNCB-sensitized	TNP-BSA	MCA2-bearer	65·5 ± 2·6*	MCA2-SF1 does not suppress LAI
TNCB-sensitized	TNP-BSA	PSA-TNCB tolerant	83·5 ± 2·7	PSA-SF1 suppresses LAI

*$P < 0·01$; other values not significantly different from control.

† Serum from mice injected with picrylsulphonic acid (PSA) and 6 days later painted with TNCB.

Table 2. Activity of SF1 in tumour-bearer serum: effect of prior treatment of antigen-reactive cells of tumour-bearing mice with anti-Ly-2·1 monoclonal antibody or anti-I-J antiserum

Treatment of PC	Antigen	Serum donor	% adherence (mean ± SE)	Interpretation
Medium + C†	—	Normal	87·0 ± 2·7	
Medium + C	MCA2 ext.	Normal	72·5 ± 2·6*	Significant LAI
Medium + C	MCA2 ext.	MCA2-bearer	83·3 ± 4·0	Suppression
Anti-Ly-2·1 + C	—	Normal	85·1 ± 2·9	
Anti-Ly-2·1 + C	MCA2 ext.	Normal	63·1 ± 1·4*	Significant LAI
Anti-Ly-2·1 + C	MCA2 ext.	MCA2-bearer	59·9 ± 3·0*	No suppression
Anti-I-J + C	—	Normal	85·9 ± 2·9	
Anti-I-J + C	MCA2 ext.	Normal	58·9 ± 3·4*	Significant LAI
Anti-I-J + C	MCA2 ext.	MCA2-bearer	51·5 ± 3·2*	No suppression

*$P < 0·01$; other values not significantly different from control.

†C, complement.

cally suppressed the activity of tumour-bearer PC reacting with tumour extract, and serum from mice rendered tolerant to TNCB (PSA-SF1) specifically suppressed only the relevant PC reaction.

The tumour-specificity or hapten-specificity of the antigens of Table 1 has been repeatedly confirmed (Noonan & Halliday, 1978; Koppi & Halliday, 1982) and no further data are given here.

When tumour-bearer PC were treated with antibodies to the Ly-2·1 and I-J surface markers, then with complement, the remaining cells retained their LAI reactivity with tumour extract but were no longer susceptible to suppression with SF1 (Table 2). This observation showed that the target cells for SF1 were Ly-2$^+$, I-J$^+$ lymphocytes, rather than the lymphokine-producing Ly-1$^+$ cells.

Production of a secondary SF

The involvement of Ly-2$^+$, I-J$^+$ cells in the action of SF1 suggested that these cells might be stimulated to produce a further secondary SF (now called SF2). Incubation of SF1 (in tumour-bearer serum) with tumour-bearer leucocytes alone did not stimulate them to form SF2 (data not shown, but this is further explored below in Table 5). However, when SC from tumour mice were treated with corresponding serum and then washed, they became armed so that the addition of antigen

Table 3. Production of a secondary SF (SF2) by incubation of tumour-bearer SC with SF1 and related tumour extract†

Production of SF2			Assay of SF2 on PC of MCA-bearing mice		
SC donor	Source of SF1	Incubation of armed SC with	Antigen	% adherence (mean ± SE)	Interpretation
—	—	—	—	91·7 ± 1·7	
—	—	—	MCA2 ext.	78·9 ± 2·6*	Significant LAI
—	—	MCA2 ext.	—	94·5 ± 2·0	
—	—	MCA2 ext.	MCA2 ext.	69·5 ± 3·5*	Significant LAI
Normal	Normal serum	MCA2 ext.	MCA2 ext.	66·4 ± 2·4*	No suppression
Normal	MCA2 serum	MCA2 ext.	MCA2 ext.	69·2 ± 3·2*	No suppression
MCA2-bearer	Normal serum	MCA2 ext.	MCA2 ext.	66·3 ± 2·7*	No suppression
MCA2-bearer	MCA2 serum	MCA2 ext.	MCA2 ext.	88·4 ± 3·0	SF2 produced

* $P < 0·01$; other values not significantly different from control.

† SC from normal and tumour-bearing CBA mice were armed by incubation with SF1 in tumour-bearer serum (or normal serum control), then further treated with tumour extract. The supernatants were assayed for SF2 by their effect on the LAI reaction (the indicator system).

Table 4. Production of SF2: effect of prior treatment of SC with anti-Ly-2·1 antibody or anti-I-J antiserum

Production of SF2 from SC† of MCA2-bearing mice			Assay of SF2 on PC of MCA2-bearing mice		
SC	Treatment of SC	Incubation of armed SC with:	Antigen	% adherence (mean ± SE)	Interpretation
–	—	—	—	84·2 ± 2·8	
–	—	—	MCA2 ext.	68·3 ± 3·6	Significant LAI
–	—	MCA2 ext.	—	85·3 ± 2·3	
–	—	MCA2 ext.	MCA2 ext.	74·9 ± 2·3*	Significant LAI
+	Medium + C	MCA2 ext.	MCA2 ext.	80·6 ± 3·2	SF2 produced
+	Anti-Ly-2·1 + C	MCA2 ext.	MCA2 ext.	64·9 ± 3·3*	No SF2 produced
+	Anti-I-J + C	MCA2 ext.	MCA2 ext.	66·7 ± 2·8*	No SF2 produced

* $P < 0·01$; other values not significantly different from control.

† All SC were first treated with medium or antibodies and complement (C). The SC were then armed with SF1 in MCA2 serum and stimulated with MCA2 extract to produce SF2.

elicited SF2, which could be assayed in supernatants by using a suitable indicator system (Table 3). SC were used as a source of SF2, rather than PC, only because greater numbers were available. The amount of tumour extract used to produce SF2 was not sufficient to give LAI in the indicator PC, as is also shown in Table 3.

When anti-Ly-2 or anti-I-J antibodies were used as cytotoxic pretreatments of SC before arming with SF1, no SF2 was produced (Table 4). This confirms the previous results of Table 2, and in addition shows that a SF is nowly formed, since the indicator system is now separated from the production system.

It seemed that 'acceptor' cells in a sensitized leucocyte population could be armed by SF1 to become antigen-reactive and produce SF2. The question of the specificity requirements of this multi-component mixture arose, and was answered by the results of Table 5. SF2 was produced only when sensitized SC reacted with the corresponding SF1 and antigen. Syngeneic TNP-cells (haptenized with PSA) were used as one of the antigens to conform more closely to experiments conducted by other workers (Zembala *et al.*, 1982b).

Table 5. Production of SF2: specificity of acceptor SC and SF1

Production of SF2			Assay of SF2 on PC of MCA2-bearing mice		
SC donor	Source of SF1	Antigen	Antigen	% adherence (mean ± SE)	Interpretation
—	—	—	—	90·3 ± 2·2	
—	—	—	MCA2 ext.	73·0 ± 1·9*	Significant LAI
MCA2-bearer	MCA2-bearer serum	MCA2 ext.	MCA2 ext.	87·5 ± 3·0	SF2 produced
MCA2-bearer	MCA2-bearer serum	TNP cells†	MCA2 ext.	69·7 ± 2·9*	SF2 not produced
MCA2-bearer	PSA-TNCB tolerant serum	MCA2 ext.	MCA2 ext.	72·4 ± 3·2*	SF2 not produced
MCA2-bearer	PSA-TNCB tolerant serum	TNP cells	MCA2 ext.	72·0 ± 2·9*	SF2 not produced
TNCB-sensitized	MCA2-bearer serum	MCA2 ext.	MCA2 ext.	71·6 ± 4·3*	SF2 not produced
TNCB-sensitized	MCA2-bearer serum	TNP cells	MCA2 ext.	78·0 ± 3·6*	SF2 not produced
TNCB-sensitized	PSA-TNCB tolerant serum	MCA2 ext.	MCA2 ext.	70·3 ± 2·5*	SF2 not produced
TNCB-sensitized	PSA-TNCB tolerant serum	TNP cells	MCA2 ext.	89·8 ± 2·1	SF2 produced

* $P < 0.01$; other values not significantly different from control.

† Normal syngeneic SC were haptenized by incubation with PSA as described in the Materials and Methods.

Table 6. Properties of SF2: effect of prior treatment of tumour-bearer indicator PC with anti-Ly-2·1 antibody or anti-I-J antiserum

Treatment of PC	Antigen	Serum	SF2†	% adherence (mean ± SE)	Interpretation
Anti-Ly-2·1 + C	—	Normal	−‡	81·0 ± 3·4	
Anti-Ly-2·1 + C	MCA2 ext.	Normal	−‡	62·1 ± 2·3*	Significant LAI
Anti-Ly-2·1 + C	MCA2 ext.	MCA2	−‡	64·3 ± 3·8*	No suppression by MCA2 serum
Anti-Ly-2·1 + C	–	Normal	−§	86·5 ± 2·4	
Anti-Ly-2·1 + C	MCA2 ext.	Normal	−§	61·1 ± 2·6*	Significant LAI
Anti-Ly-2·1 + C	MCA2 ext.	Normal	+	84·1 ± 3·9	Suppression by SF2
Anti-I-J + C	—	Normal	−‡	77·2 ± 4·4	
Anti-I-J + C	MCA2 ext.	Normal	−‡	55·0 ± 3·1*	Significant LAI
Anti-I-J + C	MCA2 ext.	MCA2	−‡	40·4 ± 2·9*	No suppression by MCA2 serum
Anti-I-J + C	—	Normal	−§	72·8 ± 3·7	
Anti-I-J + C	MCA2 ext.	Normal	−§	51·8 ± 4·7*	Significant LAI
Anti-I-J + C	MCA2 ext.	Normal	+	66·2 ± 3·9	Suppression by SF2

* $P < 0.01$; other values not significantly different from control.

† SF2 was produced by arming SC from MCA2 tumour-bearing mice with tumour-bearer serum (SF1) and further incubating with MCA2 extract.

‡ Medium only.

§ Control mixture (MCA2 extract + normal mouse serum as used for production of SF2).

Properties of SF2

Does SF2 resemble SF1 in requiring Ly-2$^+$ I-J$^+$ target cells for its action? This was investigated by preparing SF2 and adding it to an indicator system in which the PC had been treated with cytotoxic anti-Ly-2 or anti-I-J antibodies and complement. Table 6 shows that MCA2 tumour-bearer serum (SF1) had no suppressive activity in such an indicator system, indicating that the system had been suitably depleted. In contrast, SF2 suppressed LAI reactivity under these conditions, and therefore has a different site of action.

SF1 is antigen-specific and strain-restricted (Noonan & Halliday, 1980; Koppi *et al.*, 1981). SF2 has neither of these properties, as shown in Table 7. When SF2 was prepared from MCA2 tumour-bearing CBA mouse SC, it was effective in suppressing indicator systems with TNCB-sensitized PC and bladder carcinoma-sensitized PC, in CBA and BALB/c mice.

Anti-I-J antibody can be used to remove SF1 from serum, indicating the presence of an I-J molecular marker (Koppi *et al.*, 1981). This property is confirmed by the data of Table 8 which show that the suppressive activity of tumour-bearer serum (SF1) was absorbed by anti-I-J Sepharose gel but SF2 was not affected

Table 7. Properties of SF2: lack of antigen-specificity and strain-restriction†

PC donor	Antigen	SF2‡	% adherence (mean ± SE) Exp. 1	Exp. 2	Interpretation
TNCB-sensitized CBA mice	—	−	86·7 ± 2·3	88·6 ± 2·8	
TNCB-sensitized CBA mice	TNP-BSA	−	75·0 ± 1·4*	73·4 ± 2·2*	Significant LAI
TNCB-sensitized CBA mice	TNP-BSA	+	82·0 ± 3·0	92·8 ± 1·4	Suppression by SF2
TNCB-sensitized BALB/c mice	—	−	84·1 ± 2·1	66·1 ± 3·5	
TNCB-sensitized BALB/c mice	TNP-BSA	−	59·9 ± 2·1*	48·4 ± 4·7*	Significant LAI
TNCB-sensitized BALB/c mice	TNP-BSA	+	73·1 ± 3·9	67·1 ± 2·1	Suppression by SF2
Bladder-carcinoma (1660) BALB/c mice	—	−	83·4 ± 3·7	88·2 ± 3·2	
Bladder-carcinoma (1660) BALB/c mice	1660 ext.	−	69·7 ± 3·3*	73·3 ± 3·0*	Significant LAI
Bladder-carcinoma (1660) BALB/c mice	1660 ext.	+	80·5 ± 3·9	81·1 ± 4·4	Suppression by SF2

* $P < 0{\cdot}01$; other values not significantly different from control.

† PC from suitably sensitized CBA or BALB/c mice were incubated with the corresponding antigen, and the effect of SF2 on LAI was determined.

‡ SF2 was produced by arming SC from MCA2 tumour-bearing CBA mice with MCA2 tumour-bearer serum (SF1) and further incubating with MCA2 extract. Mixtures without SF2 ('−') contained the same amount of MCA2 extract.

Table 8. Properties of SF2: lack of absorption by anti-I-J antiserum

SF2†	Treatment of SF2	Assay of SF on PC of MCA2 tumour-bearing mice: Antigen	Serum	% adherence (mean ± SE)	Interpretation
−	—	—	Normal	84·6 ± 2·3	
−	—	MCA ext.	Normal	66·7 ± 2·4*	Significant LAI
−	—	MCA ext.	MCA2	81·3 ± 3·3	Suppression by SF1
−	—	MCA ext.	MCA2 (absorbed with anti-I-J)	62·0 ± 4·9*	SF1 absorbed
−	—	MCA ext.	MCA2 (absorbed with control anti-Ia)	82·0 ± 2·4	SF1 not absorbed
+	—	MCA ext.	Normal	78·1 ± 2·5	Suppression by SF2
+	Absorbed with anti-I-J antiserum	MCA ext.	Normal	78·5 ± 2·8	SF2 not absorbed
+	Absorbed with control anti-Ia antiserum	MCA ext.	Normal	82·7 ± 2·7	SF2 not absorbed

* $P < 0{\cdot}01$; other values not significantly different from control without antigen.

† SF2 was produced by arming SC from MCA2 tumour-bearing mice with MCA2 tumour-bearer serum (SF1) and further incubating with MCA2 extract.

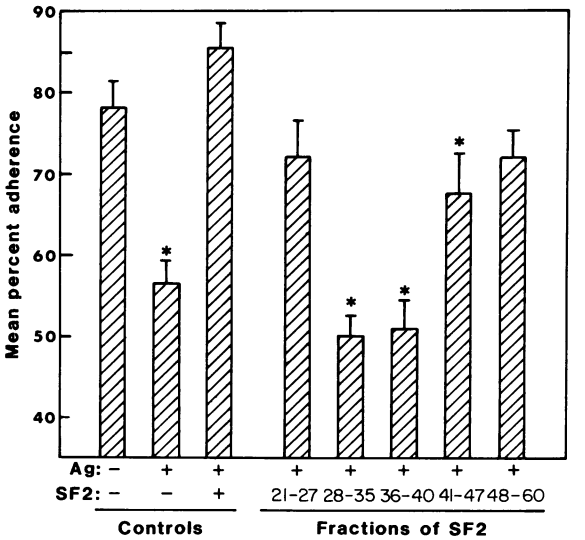

Figure 1. Suppression of LAI by secondary suppressor factor (SF2) and its Sephacryl S-200 fractions. SF2 was produced by arming SC from MCA2-bearing mice with MCA2-bearer serum (SF1) and then stimulating these armed SC with MCA2 tumour extract. The SF2 was assayed in supernatant or fractions by adding to mixtures containing PC from MCA2-bearing mice and MCA2 tumour extract as antigen (Ag). Vertical bars show standard error of the mean. *Significant LAI ($P < 0.01$).

by the same absorbing treatment. When the gel used to treat the SF2 preparation was washed and then eluted at low pH, no SF was detected in the eluate (data not shown).

SF2 was passed through the calibrated Sephacryl S-200 column and fractions were collected, pooled and assayed for suppression in LAI. The results are expressed diagrammatically for original SF2 and its fractions in Fig. 1. SF2 activity was detected in the original supernatant and in two peaks corresponding to MW > 190,000 (Fractions 21–27) and 20,000–50,000 (Fractions 48–60).

DISCUSSION

Soluble SF that interfere with the expression of CMI in an antigen-specific fashion are known from several areas of immunology, but their mode of action is obscure. Several elaborate mechanisms have been proposed (Nepom, Hellström & Hellström, 1983; Dorf & Benacerraf, 1984) to explain cell–SF interactions. Appropriately sensitized lymphoid cells are known to absorb SF from serum (Sjögren *et al.*, 1971; Phillips & Halliday, 1983), but these factors do not directly suppress the antigen reactivity of cells. The data presented in this paper indicate a more complex pathway involving intermediate cells and factors.

Mixed lymphoid cell populations from tumour-bearing or hapten-sensitized mice are normally capable of reacting with appropriate antigens, and this reaction can be suppressed by SF in serum (Hellström & Hellström, 1969; Halliday & Noonan, 1978; Koppi *et al.*, 1981). We have shown previously that sensitized Ly-1$^+$ T lymphocytes, with macrophages, are sufficient to demonstrate *in vitro* CMI against relevant tumour antigens (Koppi & Halliday, 1982). In the present study, populations depleted of Ly-2$^+$ or I-J$^+$ cells, although containing LAI-reactive lymphocytes, were found not to be susceptible to suppression by the appropriate serum SF (SF1). The Ly-2$^+$, I-J$^+$ cells were required for the production of a further SF (SF2), provided that a particular set of conditions applied. Firstly, these cells had to be derived from specifically sensitized animals. Secondly, these cells had to be 'armed' with specific SF1. Thirdly, treatment with specific antigen was required for armed cells to liberate free SF2 that could be assayed in supernatants. Similar cells have been described by Zembala *et al.* (1982b) as T acceptor cells, but are claimed to be non-specific, requiring immunization for their production but not specific immunization. Ptak *et al.* (1983) also suggested that there was no need of specifically sensitized Ly-2$^+$, I-J$^+$ cells for the production of secondary suppressor factor, as antigen priming only expanded the population of cells that served as a source of non-specific secondary suppressor factor. In addition to these differences in requirements for production, there are other distinctive properties of SF2. The secondary suppressor factor (SF2) that suppresses LAI reactions is not strain-restricted and appears in two different molecular forms (MW > 190,000 and 20,000–50,000); the secondary suppressor factor that suppresses *in vivo* reactions (Zembala *et al.*, 1982b; Ptak *et al.*, 1983) appears to be strain-restricted and only of a low MW (20,000–50,000).

Although specifically sensitized Ly-2$^+$, I-J$^+$ cells are required for SF2 production, they may act as intermediary cells in transferring the suppressor signal to yet another cell population. Macrophages have been shown to produce secondary non-specific suppressor factors (Ptak *et al.*, 1978), but the MW of macrophage-elaborated SF (10,000–20,000) is lower than SF2 (Fig. 1).

The properties of SF2 are quite distinct from those of SF1. SF2 suppresses *in vitro* reactions of CMI in a non-specific fashion and does not require a 'suppressor' cell as target; it is not strain-restricted and has a corresponding lack of the I-J marker. Both SF1 and SF2 appear to exist in two molecular forms of different MW; this has been reported previously for SF1 (Halliday *et al.*, 1982) and is shown here in Fig. 1 for SF2. The MW ranges for the components of SF1 and SF2 are similar. We have found (unpublished data) that both molecular forms of SF1 can arm sensitized SC and induce the formation of SF2 *in vitro*.

One might suggest that the SF2 described here resemble the leucocyte adherence stimulation factor that is released by sensitized leucocytes on stimulation with relevant antigen, under certain circumstances (Dunn & Halliday, 1981). We have tested SF2 for its effect on normal leucocytes and have found that it does not stimulate adherence. Whether SF2 produced as described in the present study can suppress *in vivo* reactions is not known.

The multiple factors and cells described here are reminiscent of the more familiar suppressor cascade described in great detail by other investigators (Dorf & Benacerraf, 1984; Asherson *et al.*, 1984). Some features of the present work are distinctive and may account for the novel findings. Thus, the primary SF (SF1) is a circulating factor found in serum, and the target system for suppression is a rapid *in vitro* manifestation of CMI. Although the total elapsed time required for the assay system is only 1·5 hr, there is apparently ample opportunity for a complex interplay between numerous cell types and different soluble mediators.

ACKNOWLEDGMENTS

We are indebted to Prof. I. F. C. McKenzie for the generous gift of antibodies, and Drs I. and K. E. Hellström for supplying murine tumours. This work was supported by the National Health & Medical Research Council of Australia and the Queensland Cancer Fund.

REFERENCES

ASHERSON G.L., DORF M.E., COLIZZI V., ZEMBALA M. & JAMES B.M. (1984) Equivalance of conventional anti-picryl T suppressor factor in the contact sensitivity system and monoclonal anti-NP TsF3: their final non-specific effect via the T acceptor cell. *Immunology*, **53,** 491.

DORF M.E. & BENACERRAF B. (1984) Suppressor cells and immunoregulation. *Ann. Rev. Immunol.* **2,** 127.

DUNN I.S. & HALLIDAY W.J. (1981) Inhibition and stimulation of leukocyte adherence by soluble factors from murine lymphocytes. *Immunol. Lett.* **3,** 351.

GREENE M.I., BACH B.A. & BENACERRAF B. (1979) Mechanisms of regulation of cell mediated immunity. III. The characterization of azobenzenearsonate (ABA) specific suppressor T cell derived suppressor factors (SF). *J. exp. Med.* **149,** 1069.

HALLIDAY W.J., KOPPI T.A., KHAN J.M. & DAVIS N.C. (1980) Leukocyte adherence inhibition: tumor specificity of cellular and serum blocking reactions in human melanoma, breast cancer and colorectal cancer. *J. natl. Cancer Inst.* **65,** 327.

HALLIDAY W.J., KOPPI T.A. & MCKENZIE I.F.C. (1982) Regulation of cell-mediated immunologic reactivity to Moloney murine sarcoma virus-induced tumors. III. Further characterization of tumor-specific serum blocking factors. *J. natl. Cancer Inst.* **69,** 939.

HALLIDAY W.J. & MILLER S. (1972) Leukocyte adherence inhibition: a simple test for cell-mediated tumour immunity and serum blocking factors. *Int. J. Cancer*, **9,** 477.

HALLIDAY W.J. & NOONAN F.P. (1978) Studies of contact hypersensitivity and tolerance *in vivo* and *in vitro*. II. Regulation by serum factors. *Int. Arch. Allergy appl. Immunol.* **56,** 533.

HELLSTRÖM I. & HELLSTRÖM K.E. (1969) Studies on cellular immunity and its serum-mediated inhibition in Moloney-virus-induced mouse sarcomas. *Int. J. Cancer*, **4,** 587.

HUDSON L. & HAY F.C. (1980) *Practical Immunology*, 2nd edn, p. 5. Blackwell Scientific Publications, Oxford.

KOPPI T.A. & HALLIDAY W.J. (1982) Further characterization of the cells involved in leukocyte adherence inhibition with murine tumor extracts. *Cell Immunol.* **66,** 394.

KOPPI T.A. & HALLIDAY W.J. (1983) Cellular origin of blocking factors from cultured spleen cells of tumor-bearing mice. *Cell. Immunol.* **76,** 29.

KOPPI T.A., HALLIDAY W.J. & MCKENZIE I.F.C. (1981) Regulation of cell-mediated immunologic reactivity to Moloney murine sarcoma virus-induced tumors. II. Nature of blocking and unblocking factors in serum. *J. natl. Cancer Inst.* **66,** 1097.

KOPPI-REYNOLDS T.A. & HALLIDAY W.J. (1984) A two-chain tumour-related suppressor factor specific for a sequenced antigen. *Immunol. Lett.* **8,** 219.

NEPOM G.T., HELLSTRÖM I. & HELLSTRÖM K.E. (1983) Suppressor mechanisms in tumor immunity. *Experientia*, **39,** 235.

NOONAN F.P. & HALLIDAY W.J. (1978) Studies of contact hypersensitivity and tolerance *in vivo* and *in vitro*. I. Basic characteristics of the reactions and confirmation of an immune response in tolerance mice. *Int. Arch. Allergy appl. Immunol.* **56,** 523.

NOONAN F.P. & HALLIDAY W.J. (1980) Genetic restriction of the serum factor mediating tolerance in trinitrochlorobenzene hypersensitivity. *Cell. Immunol.* **50,** 41.

PHILLIPS R.G. & HALLIDAY W.J. (1983) Detection of specific murine anti-tumour immunity and serum factors by the indirect E-rosette augmentation assay. *Aust. J. exp. Biol. Med. Sci.* **61,** 173.

POWELL A.E., SLOSS A.M. & SMITH R.N. (1978) Leukocyte adherence inhibition: a specific assay of cell-mediated immunity dependent on lymphokine-mediated collaboration between T-lymphocytes. *J. Immunol.* **120,** 1957.

PTAK W., GERSHON R.K. & FLOOD P.M. (1983) Mechanism of suppression in the transfer of contact sensitivity. *J. exp. Med.* **158,** 1822.

PTAK W., ZEMBALA M., HANCZAKOWSKA-RECKLICKA M. & ASHERSON G.L. (1978) Nonspecific macrophage suppressor factor: its role in the inhibition of contact sensitivity to picryl chloride by specific T suppressor factor. *Eur. J. Immunol.* **8,** 645.

SJÖGREN H.O., HELLSTRÖM I., BANSAL S.C. & HELLSTRÖM K.E. (1971) Suggestive evidence that the blocking antibodies of tumor-bearing individuals may be antigen-antibody complexes. *Proc. natl. Acad. Sci. U.S.A.* **68,** 1372.

ZEMBALA M., ASHERSON G.L. & COLIZZI V. (1982a) Hapten-specific T suppressor factor recognizes both hapten and I-J region products on haptenized spleen cells. *Nature (Lond.)*, **297,** 411.

ZEMBALA M.A., ASHERSON G.L., JAMES B.M.B., STEIN V.F. & WATKINS M.C. (1982b) Anti-haptene T suppressor factor acts through an $I\text{-}J^+$, Lyl^-2^+, T acceptor cell that releases a nonspecific inhibitor of the transfer of contact sensitivity when exposed to antigen. *J. Immunol.* **129,** 1823.