Immunology 1979 **38** 797

Antigen-laden cells in thoracic duct lymph. Implications for adoptive transfer experiments

E. B. BELL *Department of Experimental Pathology, Manchester University Medical School, Manchester*

Accepted for publication 19 July 1979

Summary. T cells from thoracic duct lymph of donor rats suppress the adoptive secondary response to human serum albumin (HSA). The original aim of the present investigation was to determine whether these non-immune cells have antigen-specific receptors. Thoracic duct lymphocytes (TDL) were depleted *in vivo* of antigen-specific T cells (negatively selected) by acutely injecting non-immune donors with HSA at the time of thoracic duct cannulation. Negatively selected TDL were mixed with memory cells (primed TDL from previously immunized donors) and transferred into irradiated recipients to assess whether the suppressive potential had disappeared. Paradoxically, the addition of negatively selected TDL (which were unresponsive to HSA) augmented the adoptive secondary anti-HSA response. Further study showed that the augmented response was mediated by a very small number of cells (~ 1 in 5000) laden with antigen that appeared in lymph of non-immune donors following HSA injection. These antigen-bearing cells were highly immunogenic and furthermore could overcome the effects of T suppressor cells *in vivo*. Once antigen laden cells were removed from lymph (by affinity chromatography), however, negatively selected TDL were found to inhibit the adoptive secondary response suggesting that either suppression in this model is non-specific or that antigen-specific suppressor cells are not selected out of the recirculating pool by antigen.

Correspondence: Dr E. B. Bell, Department of Experimental Pathology, Manchester University Medical School, Oxford Road, Manchester M13 9PT.

0019-2805/79/1200-0797$02.00

INTRODUCTION

It is well documented that acutely injected antigen has a dramatic effect on cells circulating in lymph (Sprent, Miller & Mitchell, 1971; Ford & Atkins, 1971; Rowley, Gowans, Atkins, Ford & Smith, 1972). Antigen-specific lymphocytes (B and T) are retained within solid lymphoid organs (Ford, 1972) creating a corresponding deficit in the recirculating pool, a phenomenon termed negative selection. The aim of the present study was to employ negatively selected TDL to assess the specificity of a suppressor cell population.

Although suppressor cells have been observed in many experimental systems *in vivo*, antigen-specific suppressors have only been elicited by provocation including such treatments as tolerance induction (Basten, Miller & Johnson, 1975; Bell & Shand, 1975a) or judicious carrier or hapten priming (Elson & Taylor, 1974; Tada & Takemori, 1974; Ishizaka & Adachi, 1976; Yamamoto, Hamaoka, Yoshizawa, Kuroki & Kitagawa, 1977). Antibody synthesis by transferred memory cells (to human serum albumin) was found to be exquisitely sensitive to a population of T cells that were obtained from the lymph of non-immune, unstimulated rats (Bell & Shand, 1975a, b; Bell & Gradwell, 1979). A detailed study by mixed cell transfer experiments (Bell & Shand, 1975a, b; Bell & Gradwell, 1979; Feldbush, 1976) demonstrated that suppression of this

adoptive secondary response was induced by Ig-negative, radiosensitive, non-immune cells that recirculated from blood to lymph (Bell & Gradwell, 1979). Whether the TDL from these virgin donors included antigen-specific suppressor cells was not known. Therefore we asked whether negatively selected TDL retained or lost their ability to inhibit the adoptive secondary response.

What we did not foresee in the experimental design was that acutely injected antigen could stimulate (or mobilize) a separate group of cells laden with antigen which then accompanied the negatively selected TDL and obscured the original aim by over-riding any effects of suppression. Part of this report documents the existence and immunogenic properties of these antigen-bearing cells and shows why it was first necessary to remove them before the question of suppressor cell specificity could be approached.

MATERIALS AND METHODS

Rats and irradiation

Inbred (AS2 × AS)F_1 and (AS2 × HO)F_1 rats (Department of Experimental Immunobiology, Wellcome Research Laboratories) were used to establish conditions under which TDL are specifically depleted of reactivity to HSA (Fig. 1). Recipients in these experiments were irradiated from a Co^{60} source as previously described (Bell & Shand, 1975a, b) and given a small inoculum of 10^7 bone marrow (BM) cells. All other experiments employed PVG/c rats from an inbred colony at Manchester University Medical School. Recipients were irradiated from a horizontally directed linear accelerator delivering 750 or 800 rad and given no BM supplement. Recipients received syngenic transferred TDL on the day of irradiation unless otherwise stated.

Antigen

Lyophilized HSA was dissolved in distilled water and employed in this soluble form (sHSA) to stimulate an adoptive secondary response. An immunogenic form of the antigen was prepared by alum precipitation (apHSA) and 1 mg of this was injected together with 8×10^9 *Bordetella pertussis* organisms (HSA-adj) in order to evoke a primary response (Bell & Shand, 1973). Bovine gammaglobulin (BGG) (Sigma Ltd) was similarly prepared in an alum precipitated form. Fresh sheep red blood cells (SRBC) were washed three to four times before injection.

Antibody determinations

Serum samples were assayed for antigen binding capacity (ABC, μg of antigen bound/ml of neat serum) and relative avidity ('s') (Celada, Schmidt & Strom, 1969) by the ammonium sulphate precipitation test (Brownstone, Mitchison & Pitt-Rivers, 1966) using ^{125}I-labelled HSA monomer as described previously (Bell & Shand, 1973; Shand & Bell, 1976). Haemagglutinating antibodies for SRBC were detected in round bottomed microtitre plates by adding 0·05 ml of a 1·5% SRBC suspension to 0·05 ml of two-fold serially diluted rat antiserum.

Cells

Lymphocytes were collected via thoracic duct fistulae as previously described (Bell & Shand, 1975a). Primed thoracic duct lymphocytes ('TDL) including memory cells were collected from donors immunized to HSA 6–46 weeks earlier. Lymphocytes negatively selected for protein antigens (Ag-TDL) were obtained from donor rats injected with specific antigen at various times before or after cannulation of the thoracic duct.

Cell fractionation

Negatively selected Ag-TDL were fractionated on affinity columns (Shand & Bell, 1976; Golstein, Wigzell, Blomgren & Svedmyr, 1972) to remove cells which might carry HSA on their surface. Washed Diakon plastic beads (I.C.I. Ltd, U.K.) were incubated with sHSA (1·5 mg of protein/ml of packed beads) at 45° for 1½ h, cooled to 4° (4 h), poured into chromatographic columns (2·5 cm diameter) and allowed to stand at 4° overnight. A 20 cm high column of coated beads was washed extensively with PBS, saturated with rat anti-HSA serum (ABC = 203; 's' = 0·83) diluted 1:3 in PBS, incubated for 1 h at 4° and extensively washed with PBS. Ag-TDL were washed three times in Dulbecco's PBS with calcium and magnesium (DAB), centrifuged through 3 ml 50% normal rat serum, resuspended to 5×10^7/ml in RPMI medium containing 1% rat serum and passed through the column at a rate of 2–3 ml/min. Cell recovery averaged 75–80% of the input.

Iodination

HSA was iodinated with carrier-free ^{125}I (Amersham) by the chloramine T method (Hunter & Greenwood, 1962). A quantity of ^{125}I-HSA was then alum precipitated for *in vivo* injection.

Tolerance induction

Tolerance to HSA was examined in detail elsewhere (Bell & Shand, 1973; Shand & Bell, 1976). Rats were injected daily, 5 days a week for 4 weeks with the following doses of sHSA (ultracentrifuged at 40,000 *g* for 2 h): 1 × 200 mg, 4 × 100 mg each week. The tolerant animals were rested 10 days before cannulating the thoracic duct.

RESULTS

Negative selection

The following experimental protocol was adopted to investigate the effect of HSA injection on recirculating lymphocytes: TDL were collected for approximately 16 h from rats bearing an indwelling thoracic duct cannula prior to injection with HSA. Pre-injection and

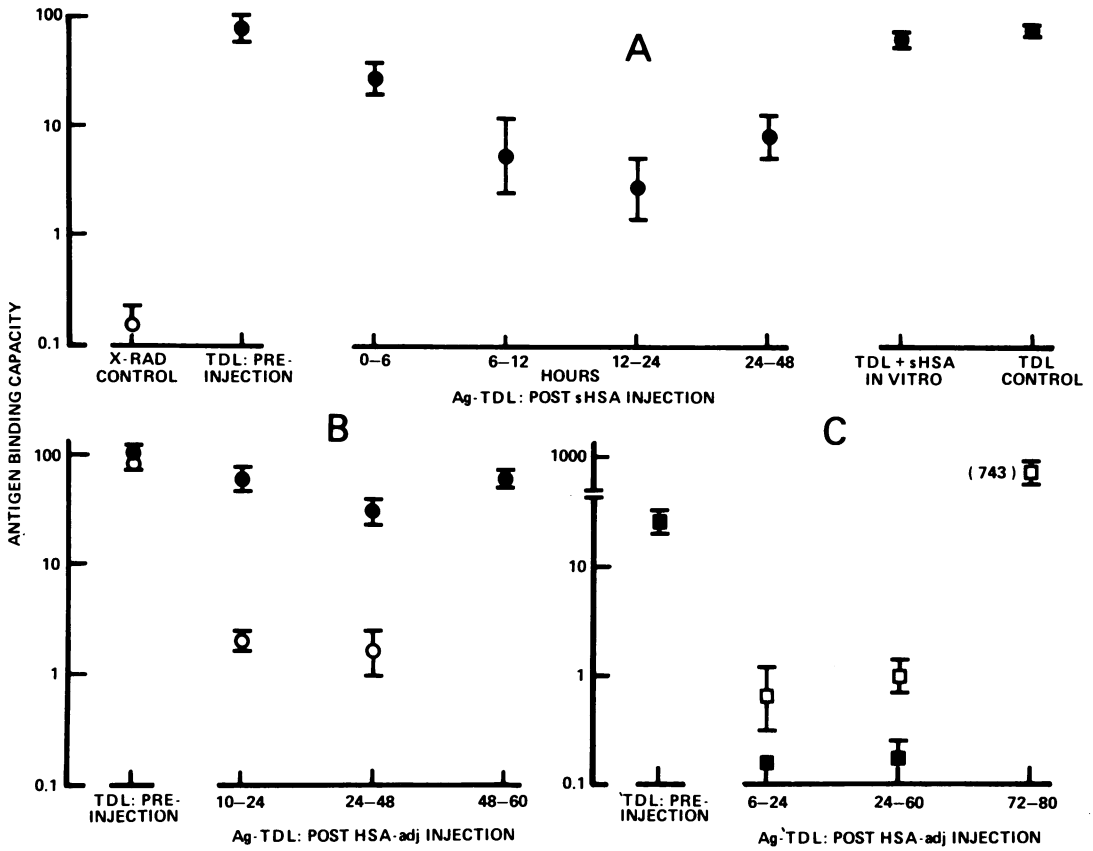

Figure 1. Depletion of HSA reactivity in TDL following injection of donors with specific antigen. (A) Negative selection with sHSA: 10^8 cells collected from donors at various times before (TDL: pre-injection) or after (Ag-TDL: post-injection) i.p. or i.v. injection of 100, 200 or 400 mg sHSA were transferred to irradiated BM restored recipients. TDL from normal uninjected donors were incubated *in vitro* at 37° for 1 h in the presence or absence of 10 mg/ml sHSA and washed before transfer. All recipients were challenged a day after cell transfer with 1 mg HSA-adj bled on day 21 and the serum tested for anti-HSA antibody. Each point represents the geometric mean (GM) ABC ± SE of six to twelve recipients (results from three experiments pooled). (B) Negative selection with HSA-adj: Ag-TDL donor rats were injected with 1 mg (•) or 10 mg (○) HSA-adj. 10^8 cells collected during different periods were transferred into irradiated BM restored recipients which were challenged the following day with 1 mg HSA-adj. Each point represents the day 21 GM ABC ± SE of two to five recipients. (C) Negative selection in HSA-primed donors: donors were immunized with HSA-adj 26 weeks before cannulating the thoracic duct; 16 h later donors were injected i.p. with 1 mg HSA-adj. $10^{7.5}$ pre-injection ('TDL) or post-injection (Ag-'TDL) were transferred to irradiated BM restored recipients which were challenged with 100 μg sHSA (■) or 100 μg HSA-adj (□) a day later. Each point represents the day 21 GM ABC ± SE of two to six recipients. The number in parentheses is the ABC value for that group.

post-injection TDL were transferred into 900 rad irradiated, syngeneic, 10^7 BM restored rats, which were challenged with 1 mg HSA-adj the following day to evoke a primary response. [A primary response in irradiated rats depends only on the transfer of helper T cells (Shand & Bell, 1976) and serum antibody is proportional to the number of specific cells transferred (Bell & Shand, 1973)]. Representative experiments from a large series are shown here to illustrate the critical features that govern negative selection of TDL for HSA and provide a rationale for later experiments.

The effect of the soluble form of HSA on recirculating cells is shown in Fig. 1A which represents the pooled results from three groups of sHSA-injected donor rats. T-cell depletion was demonstrable within 6 h of the injection of this large dose of soluble protein; smaller doses were less effective (data not shown).

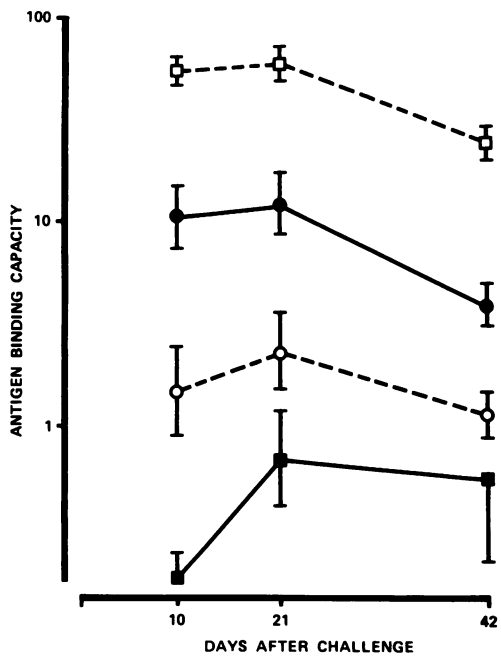

Figure 2. Augmentation of the adoptive secondary response with negatively selected TDL from donors recently injected with HSA-adj. Ag-TDL donors were injected i.p. with 10 mg HSA-adj immediately after the cannulation procedure and the cells collected 10–48 h later. 5×10^7 'TDL alone (●) or 'TDL together with 10^8 normal TDL (○) or with 10^8 Ag-TDL (□) were transferred into groups of six irradiated recipients that were challenged with 10 μg sHSA a day later. A control group of four recipients received 10^8 Ag-TDL alone (■) and were challenged the next day with 1 mg HSA-adj.

Merely exposing normal TDL to sHSA *in vitro* was not sufficient to alter its responsiveness on transfer (Fig. 1A), a result which was consistent with the experience of others (Mitchison, 1968). The use of the immunogenic form of HSA, i.e. HSA-adj was approximately ten times more efficient in depleting TDL of HSA-reactivity (Fig. 1B). A comparison between Ag-TDL selected by 10 mg and 1 mg HSA-adj also shows a clear dosage difference (Fig. 1B). Unprimed and immune rats were equally susceptible to negative selection. Primed TDL from previously immunized donors that were injected with HSA-adj after cannulation were tested in irradiated recipients for the presence of memory cells (which respond to sHSA challenge) or helper T cells capable of generating a primary response (which requires HSA-adj challenge). Between 6 and 60 h after the start of negative selection memory cells were essentially absent from lymph although some T-cell activity for a primary response remained (Fig. 1C). By 80 h, however, there was evidence of the return of memory cells. Recipients in this latter group responded vigorously on day 10 after transfer, synthesizing a large quantity (ABC = 1300) of high avidity ('s' = 0·81) antibody indicative of memory cells.

On the basis of these and other unpublished data a dose of 10 mg of alum precipitated HSA, without (apHSA) or with *B. pertussis* (HSA-adj) (which had no additional effect), was adopted for subsequent experiments that required negatively selected TDL.

Negatively selected TDL augment the adoptive secondary response

Earlier studies from this series established that the adoptive secondary response was inhibited by non-immune TDL or the T-cell fraction of non-immune TDL (Bell & Shand, 1975a, b; Bell & Gradwell, 1979). The object of the following experiment was to determine whether TDL negatively selected for HSA retained or lost their ability to suppress transferred memory cells.

Primed TDL were transferred alone or together with non-immune TDL (N-TDL) or with negatively selected TDL (Ag-TDL) into irradiated recipients which were challenged the following day with 10 μg sHSA. An additional control group receiving Ag-TDL alone was challenged with 1 mg HSA-adj to assess the effectiveness of the negative selection. The memory cell response was suppressed by N-TDL as expected (Fig. 2) but much to our surprise the addition of Ag-TDL augmented the primed TDL response. The increased

antibody synthesis by this latter group was of a similar high avidity to the control ('TDL alone) and could not have originated from negatively selected Ag-TDL itself which proved to be relatively unresponsive to the adjuvant challenge. It was therefore necessary to clarify the reasons for the augmented primed TDL response before the question of specificity of suppression could be pursued.

Was the adoptive secondary response augmented non-specifically?

It was possible that the introduction of an antigen or adjuvant into lymph donors would confer on the draining TDL adjuvant-like properties that might non-specifically stimulate memory cells on transfer. Ag-TDL donor rats were thus injected near the time of cannulation with an irrelevant antigen (SRBC). This experiment also provided a specificity control for negative slection to HSA. TDL from SRBC-injected donors inhibited the adoptive secondary response whereas TDL from HSA-injected rats again augmented the response (Table 1). A similar result was found using alum precipitated human or bovine gamma globulin as irrelevant antigens (unpublished and Table 5) and diminished the possibility that the augmentation effect was non-specific. Note that Ag-SRBC-TDL was depleted of reactivity to SRBC challenge but not to HSA (Table 1); the converse was true of Ag-HSA-TDL.

As an alternative to normal donors, HSA-tolerant rats were injected with 10 mg apHSA at the time of cannulation and provided Ag-HSA-Tol-TDL for transfer with primed TDL in the standard cell mixture protocol. It was shown previously that TDL from HSA-tolerant donors inhibit the adoptive secondary anti-HSA response to an even greater extent than normal TDL (Bell & Shand, 1975a). In contrast, however, the tolerant TDL obtained from recently challenged tolerant donors augmented the adoptive secondary response: (5×10^7 'TDL alone, day 11 ABC = 82·5; 5×10^7 'TDL + 5×10^7 AgHSA-Tol-TDL, ABC = 225·8).

The augmented adoptive secondary response is mediated by antigen-laden cells

The results suggested that specific antigen was in some way playing a direct role in augmenting the anti-HSA memory cell response. Two obvious possibilities were considered: (i) free HSA might be carried over as a contaminant in the Ag-TDL by reason of insufficient cell washing (ii) HSA might be carried by cells in the Ag-TDL population.

Table 1. The effect of Ag-TDL negatively selected for SRBC or HSA on the adoptive secondary anti-HSA response

'TDL	Additional* TDL (10^8)	Challenge†	Anti-HSA (Day 21) ABC‡	's'§	Anti-SRBC¶ Day 9	Day 21
$10^{7.5}$	None	sHSA	83·1 ± (0·14)	0·94		
$10^{7.5}$	AgSRBC-TDL	sHSA	25·0 ± (0·10)	0·90		
$10^{7.5}$	AgHSA-TDL	sHSA	161·6 ± (0·02)	0·79		
$10^{7.5}$	N-TDL	sHSA	32·2 ± (0·08)	0·90		
None	AgSRBC-TDL	HSA-adj + SRBC	87·1 ± (0·15)	0·37	3·75	3·75
None	AgHSA-TDL	HSA-adj + SRBC	37·4 ± (0·09)	0·32	7·00	6·25
None	None	HSA-adj + SRBC	0·1 ± (0·05)		< 1·0	< 1·0

* AgSRBC-TDL: negatively selected for SRBC by injecting donors with 2×10^8 SRBC i.p. the day before cannulation and i.v. immediately after. AgHSA-TDL: negatively selected for HSA by injecting donors with 10 mg apHSA i.p. the day before cannulation. N-TDL: normal TDL, uninjected donor.

† 10 μg sHSA; 1 mg HSA-adj; 2×10^8 SRBC. All challenges i.p. the day after cell transfer.

‡ Geometric mean (GM) ABC ± (SE mean $\log_{10}$ ABC) of four to six recipients per group.

§ Mean relative avidity values.

¶ Haemagglutination: $\log_2$ titre^{-1}.

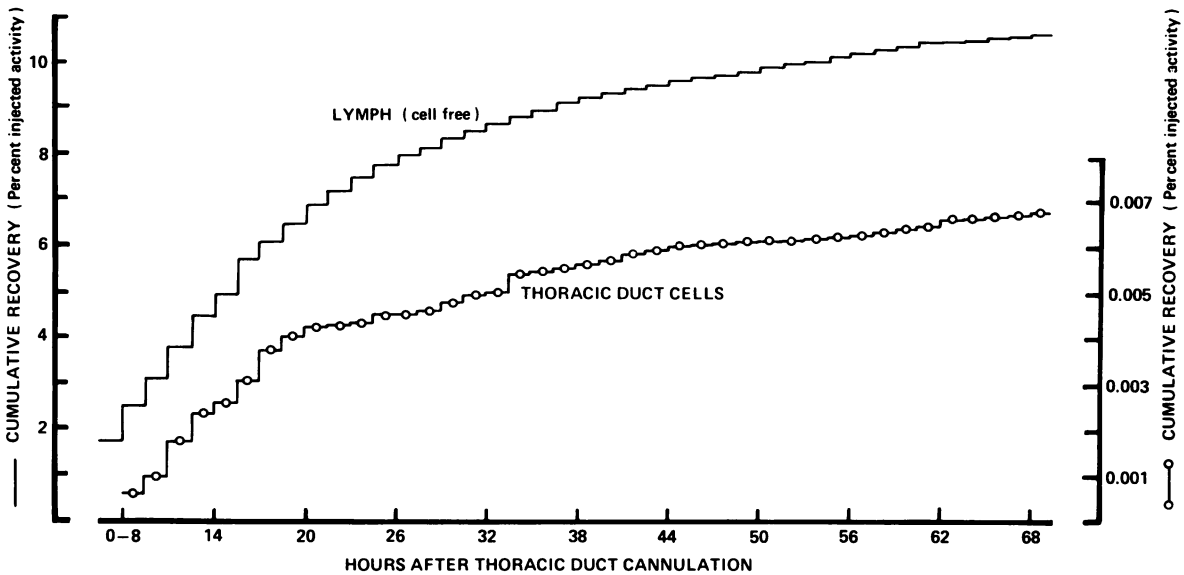

Figure 3. The appearance of radioactivity in thoracic duct lymph after i.p. injection of radiolabelled apHSA. 1 mg of ^{125}I-labelled apHSA (specific activity 28 μCi/mg) was injected immediately after cannulating the thoracic duct and 90 min fractions of lymph were collected in 1 ml Dulbecco's PBS containing 20 units of heparin.

The extent to which antigen appears free in lymph after i.p. injection of apHSA was determined using ^{125}I-labelled apHSA following injection into a thoracic duct-cannulated rat. Lymph was collected continuously (90 min intervals) on a fraction collector, centrifuged free of cells, the volume recorded and an aliquot taken for gamma counting. The cell pellet was washed extensively including a final centrifugation through a gradient of calf serum before similar counting. Radioactivity appeared in the earliest sample of lymph (Fig. 3), continued for the duration of the experiment and accounts for 10·8% of the injected dose. Two additional experiments gave very similar results. The radioactivity in cell-free lymph could be bound and precipitated by specific antibody indicating that the ^{125}I label remained conjugated to HSA but less than 0·007% of the injected dose (cummulative total, Fig. 3) was recovered in the cell pellet. This represents no more than 0·7 μg of HSA over the entire 70 h collection period.

In view of the substantial amount of free antigen in lymph that would bath the Ag-TDL during collection, further precautions were adopted to minimize potential carry-over of free antigen; Ag-TDL were washed three times in Dulbecco's PBS and centrifuged through layers of foetal calf serum or normal rat serum to remove traces of HSA.

The effect of free antigen on normal TDL was tested directly in the following experiment. The conditions of Ag-TDL collection were mimicked by adding 1 mg sHSA to the overnight flask during TDL collection from a normal donor. The cells were washed and tested in the usual way for their effect on the adoptive secondary response. The resulting antibody responses showed that collecting non-immune TDL in the presence of free antigen did not alter their capacity to inhibit primed TDL (Table 2). This differed radically from the augmenting effect of Ag-TDL where antigen was given parentally to donors (Table 2). We show elsewhere (in preparation) that TDL from donors given sHSA i.v. or i.p. is similarly able to augment primed TDL on transfer.

In a further experiment, carefully washed Ag-TDL were mixed with primed TDL, transferred into irradiated recipients but not challenged with the usual 10 μg sHSA post transfer. The results showed clearly that further challenge was not necessary (Table 3); i.e. Ag-TDL were entirely able to initiate and augment the adoptive secondary response suggesting that this effect was not associated with free antigen contaminating the Ag-TDL but more likely related in some way to the cells which were present in the thoracic duct of antigen injected donors.

Clear evidence for the existence of a small number of

Table 2. Failure of normal TDL collected in the presence of free HSA to augment the adoptive secondary anti-HSA response

'TDL	Additional TDL*	No. of recipients†	Day 21 ABC‡	Percentage of 'TDL control
$10^{7.5}$	None	6	45·1 ± (0·18)	100
$10^{7.5}$	10^8 AgHSA-TDL	8	133·5 ± (0·07)	296
$10^{7.5}$	10^8 N-TDL	6	35·0 ± (0·08)	78
$10^{7.5}$	10^8 N-TDL + HSA	6	22·0 ± (0·11)	49

* AgHSA-TDL: negatively selected for HSA by injecting donors with 10 mg HSA-adj the day before cannulation. N-TDL: normal TDL, uninjected donor. N-TDL + HSA: lymph was collected into a flask containing 1 mg sHSA.

† Recipients irradiated the day of cell transfer and challenged with 10 μg sHSA the following day.

‡ GM ABC ± (SE mean $\log_{10}$ ABC).

Table 3. Ag-TDL from donors recently injected with HSA augment the adoptive secondary anti-HSA response in the absence of further antigen challenge

'TDL	Additional TDL	No. of Recipients	Challenge	ABC* Day 8	ABC* Day 21
5×10^7	None	3	10 μg sHSA	56·3 ± (0·19)	55·5 ± (0·17)
5×10^7	None	5	None	2·6 ± (0·42)	7·0 ± (0·43)
5×10^7	10^8 Ag-TDL†	5	10 μg sHSA	147·2 ± (0·06)	66·6 ± (0·09)
5×10^7	10^8 Ag-TDL†	6	None	109·1 ± (0·06)	167·8 ± (0·05)

* GM ABC ± (SE mean $\log_{10}$ ABC)

† Obtained from donors injected with 10 mg apHSA the day before cannulation. Cells washed three times in DAB and centrifuged through a gradient of 50% normal rat serum.

cells (roughly one per 5000) in the thoracic duct, laden with antigen was obtained from an autoradiographic study (manuscript in preparation) in which Ag-TDL donors were injected with ^{125}I-apHSA. Thus it seems reasonable to conclude that antigen-bearing cells in Ag-TDL were responsible for augmenting the adoptive secondary response.

Antigen-laden cells overcome suppression

The highly immunogenic nature of Ag-TDL led us to test their functional capacity in a transfer system where suppressor cells are known to severely restrict the response of memory cells, namely non-irradiated normal recipients (Bell & Shand, 1975b; Dresser, 1961; Makela & Mitchison, 1965; Celada, 1966). The response of primed TDL in non-irradiated recipients may be inhibited by almost two orders of magnitude (Bell & Shand, 1975b) following sHSA challenge. Soluble antigen alone does not elicit a detectible response in normal rats (Bell & Shand, 1973). The results of two independent mixed cell-transfer experiments are shown in Table 4. Note that groups of rats injected with the mixture of 'TDL and Ag-TDL received no sHSA challenge. Thus the significantly increased anti-HSA response in this latter group which bear a substantial load of suppressor cells was induced by the Ag-TDL, and suggested that antigen-laden cells overcame the effects of suppressor cells. This curious pro-

Table 4. Ag-TDL from recently injected donors abrogates suppression of the adoptive secondary response in non-irradiated recipients

Expt No.	'TDL	Ag-TDL*	No. of recipients†	Challenge	Day 8 ABC‡	Day 8 's'§	Day 21 ABC	Day 21 's'
1	$7{\cdot}6\times10^7$	None	4	100 μg sHSA	0·44 ± (0·06)	NE¶	0·66 ± (0·31)	NE
	$7{\cdot}6\times10^7$	2×10^8	5	None	9·6 ± (0·20)	0·91 ± 0·01	24·0 ± (0·09)	0·94 ± 0·01
					Day 10		Day 21	
2	10^8	None	5	100 μg sHSA	0·98 ± (0·22)	0·56 ± 0·05	1·18 ± (0·14)	0·55 ± 0·01
	10^8	8×10^7	5	None	13·5 ± (0·10)	0·89 ± 0·01	31·3 ± (0·06)	0·92 ± 0·01

* Obtained from donors injected i.p. with 10 mg apHSA immediately after cannulation. Cells were washed three times in DAB and centrifuged through a layer of 50% normal rat serum before transfer.

† Non-irradiated, normal rats.

‡ GM ABC ± (SE mean $\log_{10}$ ABC).

§ Mean relative avidity ± SE.

¶ No estimate possible.

perty was likely to obscure any putative suppressor activity which might remain in negatively selected TDL. Thus it was necessary to remove antigen-laden cells from Ag-TDL before one could assess the suppressive activity of negatively selected lymphocytes.

The effect of negatively selected, antigen-laden cell-depleted TDL on the adoptive secondary response

On the likelihood that HSA would be displayed on the surface, depletion of antigen-bearing cells was attempted using affinity chromatographic columns (Shand & Bell, 1976; Golstein *et al.*, 1972) containing anti-HSA antibody as described in Materials and Methods. TDL negatively selected for HSA were passed through an affinity column and transferred into two groups of irradiated recipients, a day in advance of HSA memory cells, an option which preserves both suppressor activity (Bell & Gradwell, 1979) and antigen-laden cell function (unpublished). One group (B, Table 5) was left unchallenged as a test for the presence or depletion of antigen-laden cells. The other group was compared with recipients receiving similarly passaged TDL obtained from a control donor injected with bovine gammaglobulin (apBGG) as an irrelevant antigen. Two important findings emerged from this experiment (Table 5). Firstly, the column fractionation technique apparently removed the functional antigen-laden cells as evidenced by the failure of the passaged AgHSA-TDL (group B) to stimulate primed TDL. Secondly, having removed the antigen-laden cells, the negatively selected, fractionated lymphocytes (group C) were now able to suppress the adoptive secondary response as efficiently as the control suppressor cells (group D) (39·5% and 31·2% of the uninhibited 'TDL response respectively). That is, suppressor cell activity was still demonstrable in a cell population specifically depleted of antigen-reactive T cells.

DISCUSSION

The now classic studies by Ford & Atkins (1971), Sprent *et al.* (1971) and Rowley *et al.* (1972) demonstrated that TDL from rats or mice could be negatively selected for alloantigens, SRBC and alum precipitated proteins including hapten–protein conjugates and provided the basis for the present experimental approach. The standard procedure adopted by the latter two groups (Sprent *et al.*, 1971; Rowley *et al.*, 1972) was to inject antigen into TDL donors 24 h in advance of cannulation. It was shown here that rat TDL in fact becomes depleted of specific cells (both B and T helpers) within 6 h of antigen injection and that soluble proteins as well as particulate antigens were effective provided a sufficiently large dose of soluble antigen was injected. The decision to exploit nega-

Table 5. Negatively selected TDL inhibit the adoptive secondary response after antigen laden cells are removed

Group	TDL	Column* fractionated TDL (10^8)	Challenge†	Anti-HSA Day 21 ABC‡	's'§
A	5×10^7	None	sHSA	61·9 ± (0·10)	0·96
B	5×10^7	AgHSA-TDL	None	4·7 ± (0·16)	0·92
C	5×10^7	AgHSA-TDL	sHSA	24·5 ± (0·05)	0·93
D	5×10^7	AgBGG-TDL	sHSA	19·3 ± (0·13)	0·92
E	None	AgHSA-TDL	HSA-adj + BGG-adj	11·5 ± (0·22)	0·37
F	None	AgBGG-TDL	HSA-adj + BGG-adj	33·3 ± (0·08)	0·39
G	None	None	HSA-adj + BGG-adj	< 0·10	

* Negatively selected AgHSA-TDL and AgBGG-TDL were obtained from donors injected the day before cannulation with 10 mg apHSA or 10 mg apBGG respectively. Both types of cells were fractionated on anti-HSA affinity columns (see Materials and methods) to remove antigen-laden cells for HSA.

† 10 μg sHSA; 1 mg HSA-adj; 0·5 mg BGG-adj.

‡ GM ABC ± (SE mean $\log_{10}$ ABC) of five or six irradiated recipients per group, except group G, only four.

§ Mean relative avidity values. All SE were < 0·06 and have been omitted for clarity.

tively selected TDL as a model to test the specificity of suppression reasonably presumed a similarity between the selectability of helper and suppressor cells.

During the initial attempt to determine whether inhibition of the adoptive secondary response depended on antigen-specific T cells we looked for the loss (or not) of suppression with negatively selected Ag-TDL. The fact that Ag-TDL in combination with primed TDL gave neither of the two expected responses but instead augmented the transferred secondary response (Fig. 2) was initially puzzling. The increased synthesis of antibody could not have originated from the negatively selected TDL itself which proved to be greatly depleted of HSA reactivity when tested by a strong immunogenic challenge (HSA-adj). Neither could the augmented response be linked with carry over of free antigen since the stimulating effect was not lost following extensive washing of Ag-TDL including centrifugation through gradients of serum (Table 3); besides we know from other studies that the adoptive secondary response is relatively independent of challenge dose between 1 and 1000 μg sHSA (Bell & Shand, 1977). The augmented effect was nevertheless dependent on specific antigen since neither AgSRBC-TDL (Table 1) nor AgBGG-TDL (Table 5) were able to increase antibody synthesis by the transferred memory cells. The fact that Ag-TDL augmented the primed TDL response even when no antigen challenge was given (Table 3) suggested that antigen was being transported by cells in an extremely immunogenic form. Direct confirmation of cells in the thoracic duct, laden with antigen was obtained from an autoradiographic study reported separately (manuscript in preparation). Recent evidence indicates that these cells are phagocytic.

It should be stressed that as far as we know the generation of negatively selected TDL by antigen injection is divorced from the function of antigen-laden cells which happen to be found in the thoracic duct. That is, antigen-laden cells are not the cause of the unresponsiveness of Ag-TDL but an additional and independent phenomenon.

Why the memory cell response should be augmented by the antigen-laden cells rather than merely triggered is intriguing. In retrospect, the increased production of antibody was fortuitous, for had the

Ag-TDL + 'TDL group instead merely resembled the 'TDL group we might have wrongly concluded that suppressor cells were excluded by antigen from the recirculating pool. The amount of antigen transported in 10^8 Ag-TDL represents less than 0·0006% of the injected dose or approximately 0·05 μg, i.e. two to three orders of magnitude less than the optimal challenge dose of soluble HSA. Interestingly, the antigen-laden cell-induced response cannot be duplicated by any dose of free sHSA (Bell & Shand, 1977) which suggests several possibilities.

(i) Hyperactivation of memory cells. Lymphostimulatory factors have been isolated from activated macrophages by several groups (Gery & Waksman, 1972; Rosenstreich & Mizel, 1978; Calderon, Kiely, Lefko & Unanue, 1975). Their effect on B and T cells *in vivo*, however, remains uncertain and inhibitory effects of macrophages are also known (Calderon *et al.*, 1975; Weiss & Fitch, 1978; Allison, 1978). On the other hand the immunogenicity of macrophages (and perhaps the antigen-laden cells which they resemble) is strongly correlated with antigen presentation (Unanue, 1978; Rosenthal, 1978; Niederhuber, 1978), a function upon which T cells are particularly dependent (Rosenthal, 1978; Niederhuber, 1978; Erb & Feldman, 1975; Schwartz, Yano & Paul, 1978; Sprent, 1978).

(ii) Strategic antigen localization. Cells carrying antigen must come into intimate contact with specific B and T cells presumably within the architecture of lymphoid tissue. Antigen-laden cells could home to those compartments which would allow optimal interaction with recirculating memory cells.

(iii) Avoidance of suppressor cells. Primed animals may in fact contain a mixture of memory and suppressor cells (Yamamoto *et al.*, 1977; Okumura, Takemori, Tokuhisa & Tada, 1977) which may circulate in the thoracic duct. Hence the augmented response induced by antigen-bearing cells could represent the successful evasion of suppressor cell activity. Antigen-laden cells certainly have the ability to abrogate suppression (Table 4).

The successful attempt to remove antigen-laden cells from negatively selected TDL was based on the premise that antigen might be exposed on the cell surface. The loss of antigen triggering function after passage through affinity columns suports this view, although the experiment did not rule out the possibility that cells carrying antigen were retained non-specifically on the columns. Nevertheless, once removed, it was possible to test the original prediction that recirculating suppressor cells would disappear from lymph shortly after antigen injection in the way that helper T cells do. This thesis proved to be wrong, for negatively selected TDL to HSA were able to inhibit the adoptive secondary anti-HSA response as effectively as control TDL (Table 5). This suggests that either suppression in this model is of a non-specific nature or that suppressor cells with specificity for antigen are not selected out of the recirculating pool. We are unable to distinguish between these alternatives on the present evidence. If the latter interpretation is correct, however, it would follow that specific suppressor cells are not retained within tissue by antigen bound to macrophages in the way that helper T cells are (Rowley *et al.*, 1972; Sprent, 1978) or at least that suppressor cells bind less avidly to tissue fixed antigen.

In an earlier study the question of specific suppression was approached using an HSA tolerance model (Bell & Shand, 1975a)—a system in which helper T cells were absent at least in a functional sense (Shand & Bell, 1976). In these experiments, suppressor activity for the adoptive secondary response was actually increased following tolerance induction and appeared to be specific for the tolerogen (sHSA). Whether the same cell type is responsible for tolerance-induced suppression and non-immune suppression remains uncertain for the link between the two models is based on circumstantial evidence at present. In both the earlier and the present study, however, helper T cells clearly responded differently from suppressor cells when confronted with the same form of antigen.

Evidence is accumulating which indicates that suppressor and helper cells recognize antigen differently. Suppressor cells will bind to antigen on solid phase absorbants, e.g. petri dishes (Taniguchi & Miller, 1977) or Sephadex beads (Okumura *et al.*, 1977), but helper cells will not (Wigzell, 1970). In contrast, helper cells bind to antigen when it is presented by macrophages (Rosenthal, 1978) in association with histocompatibility determinants (Rosenthal, 1978; Niederhuber, 1978; Erb & Feldmann, 1975; Schwartz *et al.*, 1978; Sprent, 1978). Activation requirements are also different for the two cell types. Whereas soluble, unbound antigen will stimulate T suppressor cells (Basten *et al.*, 1975; Bell & Shand, 1975a; Pierres & Germain, 1978), helper cells are preferentially activated by particulate or macrophage-bound antigen (Erb & Feldmann, 1975; Pierres & Germain, 1978). Similarly, suppression of the adoptive secondary response depends on soluble antigen challenge and can be abrogated by antigen which is absorbed onto alum precipitates (Bell & Shand, 1975a), complexed with

antibody (Bell & Gradwell, 1979) or carried by cells.

The available evidence is compatible with the view that suppressor cells, in contrast to helper cells, are not selected out of the recirculating pool by antigen. It is interesting that this result would be predicted by a network theory of immune regulation (Jerne, 1974). That is, the injection of antigen would not affect the circulation of suppressor cells directed against the idiotype. As an alternative explanation, either the non-immune suppressor cells are without specificity or specific suppressor cells are not retained within lymphoid tissue by antigen.

ACKNOWLEDGMENTS

I gratefully acknowledge the fine technical assistance of Mrs Susan Gradwell and wish to thank Professor W. L. Ford. The work was supported by a grant from the British Medical Research Council.

REFERENCES

ALLISON A. C. (1978) Mechanisms by which activated macrophages inhibit lymphocyte responses. *Immunol. Rev.* **40,** 3.

BASTEN A., MILLER J.F.A.P. & JOHNSON P. (1975) T cell dependent suppression of an anti-hapten antibody response. *Transplant. Rev.* **26,** 130.

BELL E.B. & GRADWELL S. (1979) Studies on the mechanism of suppression by T cells in an adoptive secondary response. *Cell. Immunol.* **42,** 113.

BELL E.B. & SHAND F.L. (1973) Cellular events in protein tolerant inbred rats. I. The fate of thoracic duct lymphocytes and memory cells during tolerance induction to human serum albumin. *Europ. J. Immunol.* **3,** 259.

BELL E.B. & SHAND F.L. (1975a) Persisting T cells in rats tolerant of human serum albumin. The significance of 'tolerant' and non-immune T cells which preferentially restrict high affinity antibody synthesis. *Europ. J. Immunol.* **5,** 481.

BELL E.B. & SHAND F.L. (1975b) Changes in lymphocyte recirculation and liberation of the adoptive memory response from cellular regulation in irradiated recipients. *Europ. J. Immunol.* **5,** 1.

BELL E.B. & SHAND F.L. (1977) The adoptive secondary response to human serum albumin under conditions of high antigen pressure. The response of high and low avidity subsets. *Immunology,* **33,** 469.

BROWNSTONE A., MITCHISON N.A. & PITT-RIVERS R. (1966) Biological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology,* **10,** 481.

CALDERON J., KIELY J.-M., LEFKO J.L. & UNANUE E.R. (1975) The modulation of lymphocyte functions by molecules secreted by macrophages. I. Description and partial biochemical analysis. *J. exp. Med.* **142,** 151.

CELADA F. (1966) Quantitative studies of the adoptive immunological memory in mice. I. An age-dependent barrier to syngeneic transplantation. *J. exp. Med.* **124,** 1.

CELADA F., SCHMIDT D. & STROM R. (1969) Determination of avidity of anti-albumin antibodies in the mouse. Influence of the number of cells transferred on the quality of the secondary adoptive response. *Immunology,* **17,** 189.

DRESSER D.W. (1961) A study of the adoptive secondary response to a protein antigen in mice. *Proc. Roy. Soc. Lond. B.* **154,** 398.

ELSON C.J. & TAYLOR R.B. (1974) The suppressive effect of carrier priming on the response to a hapten-carrier conjugate. *Europ. J. Immunol.* **4,** 682.

ERB P. & FELDMANN M. (1975) The role of macrophages in the generation of T-helper cells. I. The requirement for macrophages in helper cell induction and characteristics of the macrophage–T cell interaction. *Cell. Immunol.* **19,** 356.

FELDBUSH T.L. (1976) Inhibition of adoptive secondary responses by lymphoid cell populations. *Cell. Immunol.* **24,** 132.

FORD W.L. (1972) The recruitment of recirculating lymphocytes in the antigenically stimulated spleen. Specific and non-specific consequences of initiating a secondary antibody response. *Clin. exp. Immunol.* **12,** 243.

FORD W.L. & ATKINS R.C. (1971) Specific unresponsiveness of recirculating lymphocytes after exposure to histocompatibility antigen in F_1 hybrid rats. *Nature (New Biol.),* **234,** 178.

GERY I. & WAKSMAN B.H. (1972) Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J. exp. Med.* **136,** 143.

GOLSTEIN P., WIGZELL H., BLOMGREN H. & SVEDMYR E.A.J. (1972) Cells mediating specific *in vitro* cytotoxicity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. *J. exp. Med.* **135,** 890.

HUNTER W.M. & GREENWOOD F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.),* **194,** 495.

ISHIZAKA K. & ADACHI T. (1976) Generation of specific helper cells and suppressor cells *in vitro* for the IgE and IgG antibody responses. *J. Immunol.* **117,** 40.

JERNE N.K. (1974) Towards a network theory of the immune system. *Ann. Immunol. (Inst. Pasteur),* **125C,** 373.

MAKELA O. & MITCHISON N.A. (1965) The role of cell number and source in adoptive immunity. *Immunology,* **8,** 539.

MITCHISON N.A. (1968) Immunological paralysis induced by brief exposure of cells to protein antigens. *Immunology,* **15,** 531.

NIEDERHUBER J.E. (1978) The role of I region gene products in macrophage–T lymphocyte interaction. *Immunol. Rev.* **40,** 28.

OKUMURA K., TAKEMORI T., TOKUHISA T. & TADA T. (1977) Specific enrichment of the suppressor T cell bearing I-J determinants. Parallel functional and serological characterizations. *J. exp. Med.* **146,** 1234.

PIERRES M. & GERMAIN R.N. (1978) Antigen-specific T cell-mediated suppression. IV. Role of macrophages in gene-

ration of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)—specific suppressor T cells in responder mouse strains. *J. Immunol.* **121,** 1306.

ROSENSTREICH D.L. & MIZEL S.B. (1978) The participation of macrophages and macrophage cell lines in the activation of T lymphocytes by mitogens. *Immunol. Rev.* **40,** 102.

ROSENTHAL A.S. (1978) Determinant selection and macrophage function in genetic control of the immune response. *Immunol. Rev.* **40,** 136.

ROWLEY D.A., GOWANS J.L., ATKINS R.C., FORD W.L. & SMITH M.E. (1972) The specific selection of recirculating lymphocytes by antigen in normal and preimmunized rats. *J. exp. Med.* **136,** 499.

SCHWARTZ R.H., YANO A. & PAUL W.E. (1978) Interaction between antigen-presenting cells and primed T lymphocytes. *Immunol. Rev.* **40,** 153.

SHAND F.L. & BELL E.B. (1976) Cellular events in protein tolerant inbred rats. III. Antigen-reactive B cells in rats tolerant to human serum albumin. *Cell. Immunol.* **21,** 20.

SPRENT J. (1978) Restricted helper function of F_1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the opposite parental strain not associated with active suppression. *J. exp. Med.* **147,** 1142.

SPRENT J., MILLER J.F.A.P. & MITCHELL G.F. (1971) Antigen-induced selective recruitment of circulating lymphocytes. *Cell. Immunol.* **2,** 171.

TADA T. & TAKEMORI T. (1974) Selective roles of thymus-derived lymphocytes in the antibody response. I. Differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. *J. exp. Med.* **140,** 239.

TANIGUCHI M. & MILLER J.F.A.P. (1977) Enrichment of specific suppressor T cells and characterization of their surface markers. *J. exp. Med.* **146,** 1450.

UNANUE E.R. (1978) The regulation of lymphocyte functions by the macrophage. *Immunol. Rev.* **40,** 227.

WEISS A. & FITCH F.W. (1978) Suppression of the plaque-forming cell response by macrophages present in the normal rat spleen. *J. Immunol.* **120,** 357.

WIGZELL H. (1970) Specific fractionation of immunocompetent cells. *Transplant Rev.* **5,** 76.

YAMAMOTO H., HAMAOKA T., YOSHIZAWA M., KUROKI M. & KITAGAWA M. (1977) Regulatory functions of hapten-reactive helper and suppressor T lymphocytes. I. Detection and characterization of hapten-reactive suppressor T-cell activity in mice immunized with hapten-isologous protein conjugate. *J. exp. Med.* **146,** 74.