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Characterization of suppressor cells in anterior chamber-associated immune deviation (ACAID) induced by soluble antigen. Evidence of two functionally and phenotypically distinct T-suppressor cell populations

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SUMMARY

A large body of information exists describing the inability of animals receiving inoculations of antigen either intravenously (i.v.) or via the anterior chamber of the eye (AC) to mount delayed hypersensitivity (DH) responses to the injected antigen. Evidence indicates that the deviant humoral and cellular immunity that follows AC and i.v. inoculations of antigen is mediated, in part, by active suppression. Because of these similarities, it has been argued that immune deviation resulting from the AC inoculation [anterior chamber-associated immune deviation (ACAID)] of antigen represents nothing more than deviant immune responses known to be induced by the i.v. inoculation of antigens. Since circumstantial evidence suggests that AC injections may have unique immune effects, we wished to test the hypothesis that AC exposure to antigen elicits a unique form of systemic immune regulation. We have studied and compared the functional and phenotypic properties of suppressor cell populations induced by AC and i.v. inoculations of a soluble antigen, bovine serum albumin (BSA). Results indicate that AC inoculations of BSA (but not i.v. inoculations) activate antigen-specific, $CD8^+$, $I\text{-}J^+$ T lymphocytes which suppress the expression of DH responses, i.e. efferent suppression. We further report that AC and i.v. injection routes both activate antigen-specific afferent suppressor cell populations which impair the inductive phase of the immune response. However, the i.v.-induced afferent suppressor cells are $CD8^+$ $I\text{-}J^+$, whereas the AC-induced afferent suppressor cells are $CD4^+$. We conclude that AC and i.v. exposures to soluble antigens are not immunologically equivalent, and that ACAID represents a uniquely regulated systemic immune response to intraocular antigens.

INTRODUCTION

Deviant systemic immune responses, characterized chiefly by impaired delayed hypersensitivity (DH), are elicited by antigens inoculated intravenously (i.v.) and into the anterior chamber of the eye (AC) (Germain & Benacerraf, 1982; Kaplan, Streilein & Stevens, 1975; Mizuno, Clark & Streilein, 1989). Because soluble molecules placed in the AC rapidly gain access to the venous circulation through the trabecular meshwork (Becker, 1962; Bill, 1975), it has been argued that injections into this site are simply i.v. injections. If this were true, then the justification for studying immune responses to antigen inoculated intracamerally would evaporate.

During the past decade, several laboratories have demonstrated that AC injection of a variety of antigens induced a common form of deviant systemic immunity, termed anterior chamber-associated immune deviation (ACAID). The response is characterized by a failure to display DH, and the spleens of animals with ACAID contain T cells that suppress DH responses in naive adoptive transfer recipients. In virtually all of the experiments to date, the antigens used have been membrane-bound—in the form of transplantation antigens (Kaplan *et al.*, 1975), as hapten-modified cell-surface molecules (Ferguson, Waldrep & Kaplan, 1987) and as surface molecules encoded by viral genes (HSV-1) (Whittum *et al.*, 1983). Recently, we reported that ACAID can also be elicited by AC injections of soluble antigens, such as bovine serum albumin (BSA) (Mizuno *et al.*, 1989). Since immune deviation that superficially resembles ACAID is generated when BSA is injected i.v., it became

Abbreviations: AC, anterior chamber of the eye; ACAID, anterior chamber-associated immune deviation; ARVO, Association for Research in Vision and Ophthalmology; BSA-CFA bovine serum albumin emulsified in complete Freund's adjuvant; DH, delayed hypersensitivity; FP, footpad; ^{125}I-BSA, ^{125}iodine-labelled bovine serum albumin; i.d., intradermal; NMS, normal mouse serum; SC, subconjunctival space of the eye; s.c. subcutaneous; T_{DH}, delayed hypersensitivity T lymphocyte;Ts_{aff}, afferent T-suppressor cell; Ts_{eff}, efferent T-suppressor cell.

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important for us to determine whether the two states of immune deviation are identical or not. A recent publication already documents that the fate of AC-injected BSA is somewhat different from the fate of BSA injected i.v. implying that differences in immune responses might also prevail between the two routes (Wilbanks & Streilein, 1989).

We therefore undertook investigations into the functional and phenotypic properties of the suppressor cell populations induced, respectively, by AC and i.v. inoculations of the soluble antigen, BSA. Our results, which form the basis of this report, indicate that AC inoculations of BSA (but not i.v. inoculations) activate antigen-specific, $CD8^+$, $I\text{-}J^+$ T lymphocytes which suppress the expression of DH responses, i.e. they are efferent suppressor cells. Moreover, AC and i.v. injection routes both activate antigen-specific afferent suppressor cell populations which impair the inductive phase of the immune response. However, the i.v. induced afferent suppressor cells are $CD8^+$, $I\text{-}J^+$, whereas the AC-induced afferent suppressor cells are $CD4^+$. We conclude that AC and i.v. exposures to soluble antigens have immunologically disparate consequences.

MATERIALS AND METHODS

Animals

Six- to 12-week-old BALB/c mice (Andervont line) were obtained from the University of Miami mouse colony. All animals were treated according to the ARVO resolution on the use of animals in research. AC and subconjunctival (SC) inoculations were carried out under sodium pentobarbital anaesthesia.

Antigen inoculations

AC inoculations of BSA were carried out as described elsewhere (Niederkorn, Streilein & Shadduck, 1981). SC inoculations were accomplished using a blunt 30-gauge needle. Anaesthetized mice received a 3-μl volume of BSA (50 μg) (Sigma, St Louis, MO) inoculated into the right eye's subconjunctival space. Fifty micrograms of BSA were dissolved in 100 μl of Hank's balanced salt solution prior to the i.v. tail vein injection. In certain experiments, mice received right hind footpad (FP) inoculations of BSA (50 μg) or ovalbumin (100 μg) (OVA; Sigma Chemical Co., St Louis, MO) emulsified 1:1 in incomplete Freund's adjuvant (IFA; Difco Laboratories Inc., Livonia, MI) or subcutaneous (s.c.)inoculations (into the dorsal neck) of BSA or OVA emulsified 1:1 in complete Fruend's adjuvant (CFA; 0·5 mg mycobacterium/ml; Difco) in a total volume of 50 μl. BSA dosage and timing were based on previous studies documenting: (i) that the minimal ACAID-inducing dose of this antigen is 50 μg BSA (Mizuno *et al.*, 1989); and (ii) that at least 5 days are required for ACAID-induction following AC inoculation of BSA (Wilbanks & Streilein, 1989).

Assessment of delayed hypersensitivity (DH)

This was accomplished as described previously (Williamson & Streilein, 1988). Briefly, groups of mice primed 7 days earlier with BSA [50 μg BSA emulsified in complete Freund's adjuvant (BSA-CFA); Difco] received intradermal (i.d.) inoculations of BSA (200 μg) into the right ear pinna. The ear swelling response was then assessed 24 and 48 hr later using a micrometer (Mitutoyo 227-101, MTI Corporation, Paramus, NJ).

Assay for efferent suppression using a local adoptive transfer (LAT)

Details of the LAT have been described in previous reports (Williamson & Streilein, 1988). Splenocytes (1×10^6) were obtained from donors primed 7 days previously with BSA (50 μg) via AC, i.v. or SC routes of inoculation. These regulators were then added to a mixture of 200 μg BSA and 1×10^6 splenocytes from mice primed 7 days earlier to BSA-CFA (containing DH effector cells). The entire suspension (10 μl volume) was then inoculated into right ear pinna of syngeneic, naive mice and ear swelling responses measured 24 and 48 hr later.

Assay for afferent suppression via IUdR incorporation, in vivo

This assay is a modification of one described previously (Moorhead, 1976). Naive mice received right hind FP inoculations of 2×10^6 splenocytes from mice pretreated 7 days earlier via AC, i.v. or SC inoculations of BSA. One hour later, an inoculum of BSA (50 μg) emulsified in IFA (BSA-IFA) was injected into the same right hind FP. After a 4-day rest, proliferation in the draining popliteal lymph nodes was assessed following an intraperitoneal injection of Iodo-deoxyuridine, 5-[^{125}I] (IUdR; NEN Research Products, Boston MA), a thymidine analogue. A proliferative response occurring in the draining popliteal node (indicative of primary immune response) was reflected by an increased radioactive content of the ipsilateral draining popliteal node compared with the contralateral non-draining popliteal node, as assessed by gamma emissions.

Assessment of popliteal lymph node gamma emission

Both draining and non-draining popliteal lymph nodes were removed from the hind limb popliteal fossae of killed mice. Each node was counted separately using a 4-channel gamma counter (LKB-Wallac Clinigamma, model 1272-004, Turku, Finland). Results are expressed in c.p.m.

Negative selection of splenocytes

This was accomplished with antibody and complement (C) as described elsewhere (Streilein & Niederkorn, 1985). Briefly, suspensions of 1×10^7 splenocytes/ml NMS (0·5%) were incubated with cytotoxic antibody solutions (rat anti-CD8, Becton-Dickinson, Mountain View, CA, 1:200; mouse anti-Thy-1.2, Cedarlane Laboratory, Hornby, Ontario, 1:1000; rat anti-CD4, Becton-Dickenson, 1:500; or anti-$I\text{-}J^d$, a gift from Dr Waltenbaugh, Northwestern Medical School, Chicago, IL, 1:1000] for 30 min on ice. After washing, cells were resuspended at 2×10^7 cells/ml RPMI+HEPES solution and incubated with rabbit complement (Pel Freeze, Brown Deer, WI; 1:25) at 37° for 30 min. Thereafter, all cell suspensions were washed and the entire procedure repeated. Complement controls were treated with rabbit complement, but received no antibody treatment.

Statistical analysis

Statistical analysis of ear swelling measurements and IUdR incorporation was accomplished using a two-tailed Student's *t*-test. All P values less than 0·05 were deemed significant. All experiments were repeated at least twice.

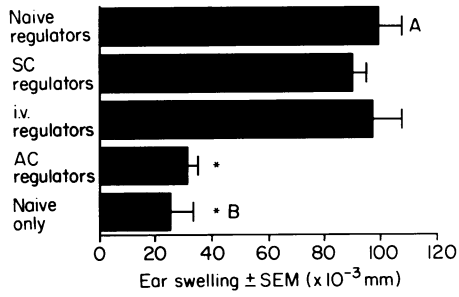

Figure 1. Description of efferent suppression following AC inoculation using a LAT. Groups of five BALB/c mice received inoculations of BSA via AC, i.v. or SC routes. Seven days later, 1×10^6 splenocytes from each group was mixed with BSA and 1×10^6 splenocytes from mice primed with BSA-CFA, s.c., 7 days earlier (DH responders). The entire suspension was then inoculated into the right ear of naive syngeneic recipients and ear swelling responses measured 24 (not shown) and 48 hr later. A positive control group (Group A) received naive splenocytes $(1 \times 10^6) +$ DH responder cells $(1 \times 10^6) +$ BSA, and a negative control group (Group B) received naive splenocytes $(2 \times 10^6) +$ BSA. Bars denote the mean ear swelling of each group ± SEM (five/group). Asterisks denote significant differences from the positive control (Group A, $P < 0{\cdot}05$).

RESULTS

Our experimental goal was to compare and contrast immune suppression elicited by i.v. and AC inoculations of the soluble antigen, BSA, and to characterize the suppressor cell population responsible for effecting ACAID. In the course of these experiments we used a local adoptive transfer (LAT) assay to detect and isolate suppression acting selectively on the efferent phase of the DH response. In this assay, splenocytes from mice pretreated with BSA via AC or i.v. routes of inoculation were co-inoculated with conventionally primed anti-BSA splenocytes. We also assessed the ability of splenocytes from donors inoculated with BSA via AC or i.v. routes to suppress the afferent or inductive limb of the immune response by injecting these cells into footpads of naive mice and then measuring the proliferative responses of popliteal lymph node lymphocytes immunized via the same footpads with BSA emulsified in incomplete Freund's adjuvant (IFA).

Description of efferent suppression using a LAT reaction

Groups of five BALB/c mice received inoculations of BSA (50 μg) via AC, i.v. or SC routes. Seven days later, the spleens of all groups (AC, i.v., SC and naive) were harvested and rendered into single cell suspensions. 1×10^6 cells from each group were mixed with BSA (200 μg) and an equal number of splenocytes from mice immunized with BSA-CFA, subcutaneously (s.c.), 7 days earlier (containing DH effector cell populations). The entire suspension (10 μl volume) was then inoculated into the intradermal (i.d.) space of the right ears of syngeneic, naive mice (five/group). Ear swelling responses were measured 24 and 48 hr later. A positive control group (Group A) received 1×10^6 naive splenocytes $+ 1 \times 10^6$ DH effector cells + BSA (200 μg). An additional group, serving as the negative control (Group B), received a right ear inoculation of 2×10^6 naive splenocytes (no primed DH effector cells) suspended in a solution containing 200 μg BSA. Results, depicted in Fig. 1, demonstrate that splenocytes only from AC-pretreated mice were able to suppress the DH response of primed immune effector cells. Functionally significant suppressor cell populations (capable of suppressing the DH response to BSA) were not found in the spleens of i.v. or SC pretreated mice, or in the positive control. As expected, mice inoculated with BSA via SC or i.v. routes, but not subsequently immunized with BSA-CFA, were devoid of DH reactivity (data not shown). Thus, in contrast to splenocytes from i.v. or SC pretreated, as well as, naive mice, splenocytes from AC pretreated mice contained suppressor cell populations capable of suppressing the efferent limb of the DH response to BSA. This finding is in agreement with results obtained by others (Streilein & Niederkorn, 1985; Waldrep & Kaplan, 1983) using P815 cells and TNP-haptenated splenocytes, respectively, and demonstrates a significant difference between the immune deviation resulting from AC and i.v. routes of antigen administration.

Description of surface markers on efferent suppressor cells from AC-primed mice

Others have demonstrated that efferent suppressor cells (Ts_{eff}) primed via the AC route of inoculation are T lymphocytes expressing CD8 and I-J surface antigens (Streilein & Niederkorn, 1985). Because these experiments were undertaken in mice immunized with P815 mastocytoma cells, which express non-water-soluble, membrane-associated antigens and because different methods were used to identify these cells, we felt it necessary to describe the surface phenotype of the suppressor cells elicited by soluble BSA.

Groups of five mice each received an AC inoculation of BSA. Seven days later their spleens were processed into single cell suspensions and treated twice with anti-CD8 + complement (C), anti-Thy-1 + C, anti-CD4 + C, anti-I-J + C, or with C alone. After treatment, 1×10^6 splenocytes were mixed with an equal number of splenocytes from mice primed 7 days earlier via s.c. injection of BSA-CFA (DH effectors). The mixture, plus 200 μg BSA, was inoculated into the ears of naive syngeneic recipients (five mice/group). A positive control group (Group A) received a mixture of naive splenocytes + DH effector cells + BSA and a negative control group (Group B) received a mixture of naive splenocytes + BSA. The results, depicted in Fig. 2, demonstrate that treatment of AC pretreated cells with anti-Thy-1 + C, anti-I-J + C or anti-CD8 + C robbed them of their capacity to suppress the DH response. Treatment with C alone or with anti-CD4 + C had no effect. Thus, Ts_{eff} cells evoked by AC inoculation of BSA display Thy-1, CD8 and I-J surface markers.

Determination of the antigen-specificity of Ts_{eff} cells

Waldrep *et al.* (1983) have previously reported that Ts_{eff} cells activated by AC inoculation of TNP-haptenated lymphocytes suppress in an antigen-specific manner. To address this issue with respect to a soluble antigen, we employed a variation of the LAT assay. Groups of five mice each received AC or SC inoculations of BSA (50 μg). Seven days later, their spleens were harvested and rendered into single cell suspensions. 1×10^6 cells were mixed with an equal number of DH effector cells from the spleens of mice immunized s.c. 7 days previously with either BSA-CFA or ovalbumin-CFA (OVA-CFA), plus 200 μg BSA or OVA, respectively. The entire mixture (10 μl volume) was then inoculated into the ears of naive, syngeneic recipients, and the ear swelling responses assessed 24 and 48 hr later. The results

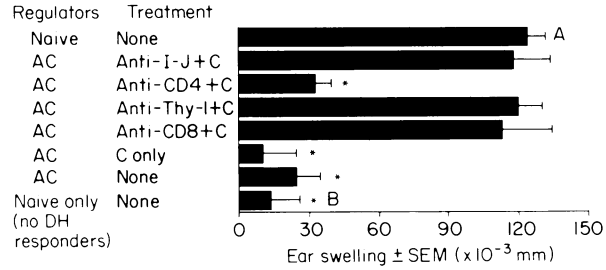

Figure 2. Determination of the surface phenotype of efferent suppressor cells from ACAID mice by negative selection with antibody+C. Groups of five mice received inoculations of BSA via the AC route. Seven days later, their splenocytes were harvested and treated twice with antibody+C or with C alone (C control). After treatment, 1×10^6 splenocytes were mixed with BSA + 1×10^6 splenocytes from mice primed 7 days earlier via s.c. inoculation of BSA-CFA (DH responders). The entire mixture (10-μl) was then inoculated into the right ear of naive syngeneic recipients and ear swelling assessed 24 (not shown) and 48 hr later. A positive control group (Group A) received naive splenocytes (1×10^6) + DH responder cells (1×10^6) + BSA, and a negative control group (Group B) received naive splenocytes (2×10^6) + BSA. Bars denote the mean ear swelling of each group ± SEM (five group). Asterisks denote significant differences from the positive control (group A, $P < 0{\cdot}05$).

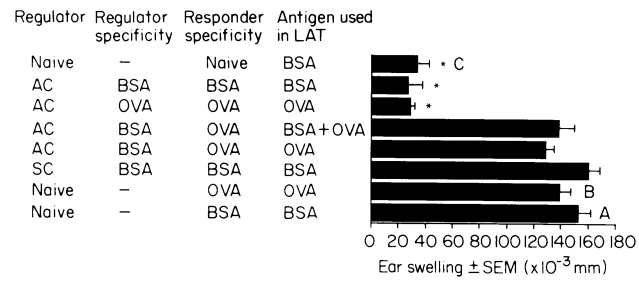

Figure 3. Determination of the antigen specificity of efferent suppressor cells from ACAID mice. Groups of five mice received inoculations of BSA or OVA via the AC or SC route. Seven days later, 1×10^6 splenocytes from these mice were mixed with BSA or OVA, plus an equal number of DH responder cells from mice primed, s.c., 7 days earlier with either BSA-CFA or OVA-CFA, respectively. The entire mixture (10 μl) was then inoculated into the ears of naive syngeneic recipients and ear swelling responses measured 24 (not shown) and 48 hr later. Positive control groups received naive splenocytes plus: BSA + BSA responders, or OVA + OVA responders (Groups A and B, respectively). A negative control group received naive splenocytes + BSA (Group C). Bars denote the mean ear swelling of each group ± SEM (five group). Asterisks denote significant differences from the positive controls (Groups A and B, $P < 0{\cdot}05$).

are depicted in Fig. 3 and reveal: (i) splenocytes exposed to BSA via AC inoculations significantly suppressed the DH response of BSA-specific immune effector cells to BSA; (ii) splenocytes exposed to OVA via AC inoculations significantly suppressed the DH response of OVA-specific immune effectors to OVA; (iii) splenocytes from AC-BSA inoculated donors were unable to suppress the DH response of OVA-specific immune effector cells to OVA; (iv) splenocytes exposed to BSA via AC inoculation were not able to suppress the DH response of OVA-specific immune effector cells to OVA, even if they were concomitantly reactivated with BSA; and (v) neither naive nor splenocytes exposed to BSA via SC inoculation were able to impair the DH response of BSA-specific immune effector cells. Thus, activation of Ts_{eff} cells is both antigen specific and antigen dependent. Moreover, even if reactivated with BSA, Ts_{eff} cells can only suppress the DH response of immune effector cells in an antigen-specific manner.

Description of afferent suppression in AC and i.v. pretreated mice

Assays of afferent suppression are difficult to devise. Previous reports that i.v. injections of soluble antigen induce afferent suppression came to that conclusion by exclusion, i.e. the spleens of i.v.-injected mice did not contain efferent suppressor cells (Ferguson *et al.*, 1987; Miller, Butler & Claman, 1982; Monroe *et al.*, 1984; Wetzig, Foster & Greene, 1982). Thus, since suppression exists, it must be afferent. In ACAID, the presence of efferent suppressors makes the prospect of detecting afferent suppressor cells even more difficult. To address this point, we chose to assess afferent suppression by means of an earlier event in the immune response – the primary proliferative response occurring in the draining lymph node of a naive mouse to inoculated antigen. Groups of five naive BALB/c mice received right hind FP inoculations of 2×10^6 splenocytes from mice inoculated with BSA 7 days earlier via AC, i.v. or SC (Group A) routes. One hour later, a second right hind FP inoculation of BSA (50 μg) emulsified in incomplete Freund's adjuvant (BSA-IFA) was inoculated into the same right hind FP. The decision to employ IFA in lieu of CFA was based on a desire to avoid false positive results associated with the primary immune response to the mycobacterium in CFA (preliminary experiments demonstrate that BSA-IFA is a suitable substitute for BSA-CFA when immunizing for DH responses; data not shown). A fourth group of mice received 2×10^6 splenocytes from naive mice followed by 50 μg BSA-IFA (Group B), and a final group received the BSA-IFA emulsion alone (Group C). These last three groups (Groups A, B and C) served as positive controls. Two more groups served as negative controls: one receiving a right hind FP inoculation of 2×10^6 naive splenocytes followed by injection of an isotonic salt solution (HBSS) emulsified in IFA (HBSS-IFA) (Group F), and a second receiving a FP inoculation of HBSS-IFA only (Group G). Four days later all mice received an i.p. inoculation of iododeoxyuridine, 5-[^{125}I] (IUdR), a thymidine analogue, which is incorporated into replicating DNA. After 5 hr, all mice were killed and the draining ipsilateral and non-draining contralateral popliteal lymph nodes (LN) were harvested and assessed separately for IUdR incorporation. The results, expressed as the ratio of ipsilateral draining LN c.p.m. to contralateral non-draining LN c.p.m. are shown in Fig. 4. Splenocytes from i.v. and from AC pretreated mice (Groups D and E, respectively) significantly suppressed the primary proliferative response to BSA-IFA in the naive recipients. By contrast, all three positive contol groups (A, B and C) mounted significant primary proliferative responses to BSA-IFA (reflecting a primary immune response), demonstrating that neither naive nor SC primed splenocytes are capable of suppressing the afferent limb of the DH response. The negative control groups (F and G) mounted only a meagre proliferative response, which is most likely due to non-specific inflammation induced by IFA. Thus, splenocytes from i.v. and AC pretreated mice are capable of suppressing the afferent limb of the anti-BSA immune response.

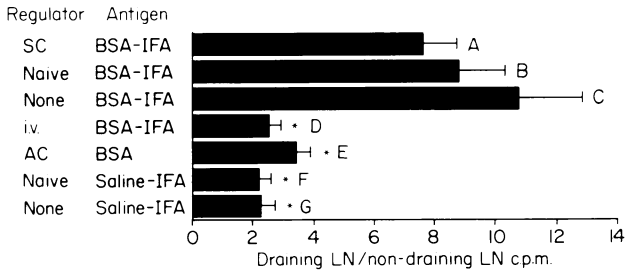

Figure 4. Documentation of afferent suppression in AC and i.v. primed mice using IUdR incorporation. Groups of five naive mice received right hind FP inoculations of 2×10^6 splenocytes from mice inoculated with BSA via AC, i.v. or SC (Group A) routes 7 days earlier. One hour later, an inoculum of BSA-IFA was administered into the same right hind FP. A fourth group received 2×10^6 naive splenocytes + BSA-IFA (Group B) and a fifth group received BSA-IFA only (Group C). These last three groups (Groups A, B and C) served as positive controls. Two groups served as negative controls: one received HBSS emulsified in IFA only (HBSS-IFA, Group G) and a second group received HBSS-IFA + 2×10^6 naive splenocytes (Group F). After a 4-day rest, all mice received an i.p. inoculation of IUdR. Five hours later, both draining and non-draining popliteal LN were harvested and assessed separately for gamma emissions. Results are expressed as the ratio of draining to non-draining LN c.p.m. Bars represent the mean of five mice group ± SEM. Asterisks denote significant differences from the positive controls (Groups A, B and C, $P < 0.05$).

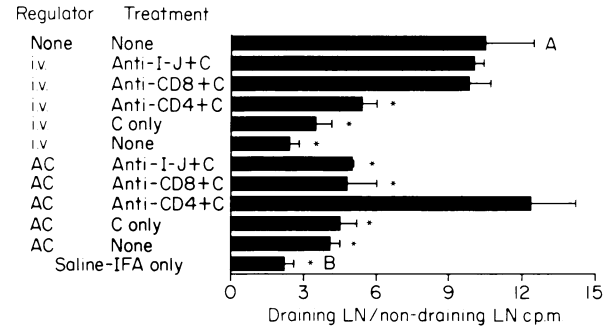

Figure 5. Description of the surface phenotype of afferent suppressor cells generated following AC and i.v. inoculations by negative selection with antibody + C. Groups of 5 mice each received right hind FP inoculations of BSA-IFA (positive control, Group A), mixed with i.v. or AC pretreated splenocytes (untreated control), or with i.v. or AC pretreated splenocytes treated twice with C alone (C control) or antibody + C. A final group served as negative control, receiving a FP inoculum of HBSS-IFA only (Group B). After a 4-day rest, cellular proliferation in both draining and non-draining popliteal LN was assessed by IUdR incorporation. Results are expressed as the ratio of draining to non-draining LN c.p.m. Bars represent the mean of five mice/group ± SEM. Asterisks denote significant differences from the positive control (Group A, $P < 0.05$).

Description of surface markers on afferent T-suppressor cells from AC and i.v. pretreated mice

We next attempted to characterize the surface phenotype of the afferent T-suppressor (Ts_{aff}) cells elicited by AC and i.v. inoculations of BSA through negative selection using specific antibodies plus C. Groups of five mice each received a right hind FP inoculation of 50 μg BSA IFA alone (positive control, Group A), mixed with i.v. or AC pretreated splenocytes or with i.v. or AC pretreated splenocytes treated with C alone, anti-I-J + C, anti-CD8 + C, or anti-CD4 + C. A final group, serving as negative controls (Group B), received a right hind FP inoculation of HBSS-IFA.

As revealed in Fig 5, treatment of AC pretreated splenocytes with anti-I-J + C or anti-CD8 + C had no affect on the ability of these cells to suppress the primary proliferative response to BSA; whereas, treatment with anti-CD4 + C did. Treatment of i.v. pretreated splenocytes with anti-I-J + C or anti-CD8 + C, but not anti-CD4 + C, abolished their ability to suppress the primary proliferative response to BSA-IFA. This indicates that i.v. pretreated Ts_{aff} cells are $CD8^-$, $I\text{-}J^+$, whereas the Ts_{aff} cells associated with AC inoculations are $CD4^+$.

Determination of the antigen specificity of Ts_{aff} cells

Groups of five mice received right hind FP inoculations (50 μl volume) of 2×10^6 splenocytes from mice inoculated with BSA via AC or i.v. routes 7 days earlier. Fifty micrograms of BSA-IFA or 100 μg of OVA-IFA were then inoculated into the same right hind FP 1 hr later (preliminary experiments demonstrate that 100 μg OVA induce a primary proliferative response equivalent to 50 μg BSA, hence the decision to inoculate with 100 μg OVA). Four days later, the primary proliferative response present in the draining popliteal lymph nodes was assessed by IUdR incorporation. We found (Fig. 6) that Ts_{aff} cells pretreated with BSA via the i.v. route suppressed the primary immune response to BSA and not OVA. Likewise, AC pretreated Ts_{aff} cells impaired the primary proliferative response to BSA in an antigen-specific manner, indicating that both inoculation routes induce antigen-specific afferent suppressor cells.

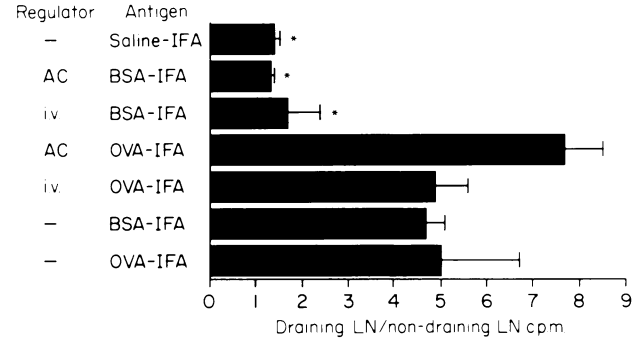

Figure 6. Determination of the antigen-specificity of afferent suppressor cells induced by AC or i.v. inoculation of BSA. Groups of five mice received right hind FP inoculations (50 μl volume) of 2×10^6 splenocytes pretreated with BSA via AC or i.v. routes 7 days earlier. 50 μg of BSA-IFA or 100 g of OVA-IFA (irrelevant antigen) were also inoculated 1 hr later. Four days later, the primary proliferative response present in the draining popliteal lymph nodes was assessed by IUdR incorporation. Results are expressed as the ratio of draining to non-draining LN c.p.m. Bars represent the mean of five mice group ± SEM. Asterisks denote significant differences from the positive control (BSA-IFA + no regulators, $P < 0.05$).

DISCUSSION

This report describes investigations into the functional and phenotypic properties of Ts cell populations induced by AC and

i.v. inoculations of soluble antigen. We demonstrate that suppression of DH followng AC and i.v. inoculations of soluble antigen is accomplished by different cell populations. Antigen-specific efferent suppression, mediated by $CD8^+$, $I\text{-}J^+$ T-lymphocytes, is generated only following AC inoculation of soluble antigen (Figs 1, 2 and 3). No efferent suppression is induced following i.v. inoculation. Thus, AC, not i.v., inoculations of BSA induce populations of Ts cells potentially capable of suppressing an ongoing DH initiated inflammatory response in an antigen-specific manner. Additionally, although antigen-specific afferent suppression of DH is present following both AC and i.v. inoculations, the cells mediating this suppression represent two different populations: i.v.-induced Ts_{aff} are $CD8^+$, $I\text{-}J^+$ whereas ACAID Ts_{aff} are $CD4^+$ (Figs 4, 5 and 6). Thus AC and i.v. immunizations induce different suppressor cell populations, implying that the mechanisms involved in mediating suppression are equally different.

Although these studies were conducted with a soluble antigen (BSA), the phenotype of the efferent suppressor cell induced by AC injection is identical to the efferent suppressors reported by others who injected membrane-bound antigens into the AC. Wetzig *et al.* (1982), Waldrep & Kaplan (1983), Streilein & Niederkorn (1985) and Ferguson *et al.* (1987) agree that the efferent suppressor cell in ACAID is $CD8^+$ and antigen specific. The fact that all of these investigators have reported the existence of such cells after AC injections of antigen, and that no such cell is generated by i.v. injection of antigen, is strong evidence in support of the contention that the AC and i.v. routes of antigen administration have distinctly different immunological consequences, at least with respect to the regulation of expression of DH. Whether these cells elaborate soluble antigen-specific suppressor factors (Liew, 1982; Zembala, Asherson & Colizzi, 1982; Zembala *et al.*, 1986) or suppress through direct cell–cell contact involving antigen-specific recognition or anti-idiotypic recognition is currently under investigation.

The surface phenotype of the afferent suppressor cells we have described in AC recipients of BSA not only differs from the i.v.-induced afferent suppressor, but from the afferent suppressors originally described by Waldrep & Kaplan (1983). We suspect that the differences reported between the TNP-induced and BSA-induced ACAID suppressor may be a reflection of the different manner in which suppressor activity was assayed. We believe that the chief value of the lymph node proliferation assay of afferent suppression is that it permits analysis without concern that the efferent suppressor cells are operative. Although we favour this explanation, other explanations may apply. For example, soluble antigens might induce a different suppressor cell network than cell-associated antigens. If that were the case, then the surface phenotype of the two afferent suppressors might be very different.

Although we are the first to formally demonstrate $CD4^+$ Ts_{aff} in ACAID animals, Heuer *et al.* (1982) have previously noted $CD8^+$ Ts_{aff} in i.v. recipients of BSA. Interestingly, these cells exerted their suppressive effect by killing $CD4^+$ T lymphocytes in an antigen-specific manner. Contrary to i.v.-induced afferent suppression, afferent suppression induced by AC inoculation of BSA is mediated by $CD4^+$ T lymphocytes. Although the mechanism of suppression by these Ts_{aff} remains unknown, one may presume it to be mediated by direct cell–cell interactions, as might occur in the case of $CD4^+$CTL directed against processed antigen bound to class II molecules on antigen-presenting cells. Suppression would occur because the antigen-presenting cells are killed. Alternatively, ACAID Ts_{aff} may elaborate soluble factors which down-regulate primary immune responses to a given antigen. Recent work in our laboratory suggests that interleukin-2 (IL-2), interferon-gamma (IFN-γ) secreting, but not IL-4- IL-5-secreting, Th cells are down-regulated in the spleen of ACAID mice (Wilbanks & Streilein, 1990). This finding, combined with *in vitro* data demonstrating that Th cell clones known as Th2 (IL-4, IL-5-secreting; Mosmann *et al.*, 1986, Mosmann & Coffman, 1987) secrete a soluble factor capable of suppressing Th1 clones (secrete IL-2, IFN-γ; Mosmann *et al.*, 1986, Mosmann & Coffman, 1987), suggests that a Th2-like cell may be preferentially activated in ACAID. According to this view, Th2 cells, once activated by antigen, impair the activation and clonal expansion of Th1-like cells that are required to mediate DH responses to that antigen (Horowitz *et al.*, 1986). If this is true, one would predict that BSA-pretreated Ts_{aff} would be capable of impairing a primary immune response to an irrelevant antigen, e.g. OVA, if BSA was concomitantly inoculated with the OVA. Experiments to test this possibility are underway.

It will also be of interest to understand what role the different types of suppressor cells induced in ACAID play in ocular immune responses. We are particularly interested in the efferent suppressor cell population. Based on teleological reasoning, we have proposed that the existence of ACAID is related to the vulnerability of the internal structures of the eye to the side-effects of DH reactions. The efferent suppressor T cell of ACAID undoubtedly acts to prevent the expression of DH in peripheral tissues, i.e. dermis of the ear. It remains to be determined whether this suppressor cell can equally well quench DH responses elicited in the anterior segment of the eye, and thereby preserve the anatomic integrity of the visual apparatus.

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