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In vitro responses to the liver antigen F

G. H. SUNSHINE, MURIEL CYRUS* & GUIL WINCHESTER† *Imperial Cancer Research Fund Tumour Immunology Unit, University College London, London and *Department of Pathology, Yale School of Medicine, New Haven, Connecticut, U.S.A.*

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Summary. In this paper we describe the first *in vitro* response to the liver alloantigen F. The anti-F response serves as a valuable model for autoimmune phenomena since priming appropriate strains of mice (responders) with allogeneic but not syngeneic type F leads to autoantibody production. The *in vitro* system is based on the proliferation of T cells, from mice primed *in vivo* with F, when coincubated with splenic adherent cells (SAC) prepulsed with F *in vivo*. The system displays two important correlates of the *in vivo* antibody response to F:1.T cells from mice primed with syngeneic F do not proliferate when incubated with SAC prepulsed with syngeneic F and 2. Mice that do not make antibody responses to allo F *in vivo* (DBA/2) do not show *in vitro* proliferative responses. These findings indicate that the proliferative assay is a good *in vitro* model for the F response.

Abbreviations: DTH, delayed type hypersensitivity; EDTA, ethylenediamine tetraacetic acid; FcR, receptor for Fc region of immunoglobulin; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; ^{125}IUdR, ^{125}Iododeoxyuridine; PPD, purified protein derivative of tuberculin; SAC, spleen cell adherent at 2 hr to microexudate-coated petri dish.

† Present address: Department of Biochemistry, Imperial College London, London.

Correspondence: Dr G. H. Sunshine, Imperial Cancer Research Fund Tumour Immunity Unit, University College, Gower Street, London W.C.1.

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INTRODUCTION

The cytoplasmic liver protein F has been extensively studied as a model for understanding how the immune response to autoantigens is regulated. The 40,000 mol.wt protein (Lane & Silver 1976) is ubiquitous in mammals; and in inbred strains of mice is found in two forms, F^1 and F^2. These can be distinguished on the basis of size and charge and represent allelic variants (Winchester, Lane & Taylor, manuscript in preparation; Anders, personal communication).

Interest in the F system originated in the fact that in certain strains of mice alloimmunization leads to an autoantibody response directed against the common determinants of the F molecule (Fravi & Lindenmann, 1968). This is interpreted as an example of the cross reactive termination of humoral self tolerance in which T-helper cells recognizing the allo determinant are able to trigger autoreactive B cells. This implies that self tolerance to the F molecule is normally maintained by T cells (Iverson & Lindenmann, 1972).

The genetics of the ability to respond to alloimmunization are also interesting. Control is polygenic, since although there is an absolute requirement for the k allele at H-2K or I-A other non-H-2 linked Ir genes are involved (Silver & Lane, 1981). In a cross between non-responder and responder strains, where non-responsiveness is dominant, F_1 mice can be converted to responders by thymectomy. The role of the T cell in both systems therefore appears central.

Detailed investigation of interactions involved in T-cell reactivity to F has been hampered, however, by the lack of an *in vitro* assay. An earlier attempt to develop a proliferation assay foundered when it was discovered that purified F was a potent inhibitor of concanavalin A-induced proliferation of lymphocytes (Lane & Silver, 1977). The effect appeared not to be due to simple toxicity nor to competition for mitogen binding sites, and its was suggested that F might be identical to the biochemically similar inhibitor of lymphocyte proliferation isolated from liver (Schumacher, Maerker-Alzer & Wehmer, 1974).

In this paper we report the first *in vitro* assay specific for F. T cells from F-primed animals are coincubated with spleen adherent cells pulsed with F *in vivo*. T-cell proliferation is then assessed at 96 hr by the incorporation of [^{125}I]ododeoxyuridine ([^{125}I]UdR) into DNA. Suppression by F is also found in this system but is circumvented by ensuring that no free antigen is present at the beginning of the culture with T cells. This assay is already proving a powerful tool for the dissection of the immune response to F antigen.

MATERIALS AND METHODS

Mice

All mice were bred at the Imperial Cancer Research Fund Colony at Mill Hill, London. AKR or CBA mice were used as a source of F^1 and mice with a B10 background were used in the preparation of F^2.

In terms of antibody response in F^1 type mice AKR and CBA strains are high responders to F^2 whereas DBA/2 do not respond. B10.Br mice are F^2 type and make an anti-F^1 antibody response (Silver & Lane 1977).

Antigens

F was prepared from mouse liver by a modification of the previously described method (Lane & Silver 1976). Briefly, mouse liver was homogenized in ice-cold PBSA at a 1:1 ratio (w/w). The cytoplasmic fraction was obtained by centrifugation at 100,000 *g* for 1 hr after removing nuclei and mitochondria. It was then precipitated with ammonium sulphate to a final concentration of 34%. The supernatant was then adjusted to make 70% ammonium sulphate. The precipitate was resuspended in 1 mM magnesium chloride, 500 mM sodium chloride and 50 mM Tris hydrochloride pH 8·5 and dialysed exhaustively against this buffer. This preparation was used as crude F. It was then run over a Sephacryl S200 column. Column fractions that reacted with standard anti-F antisera in Ouchterlony plates were pooled, dialysed against 0·01 M sodium acetate buffer pH 5·5 and run over a carboxymethyl cellulose column which had been equilibrated with acetate buffer. Elution with a linear salt gradient (0·05–0·01 M Na^+) resulted in two major protein peaks only one of which reacted with anti-F sera. This material was used as pure F antigen for these studies.

Purified protein derivative of tuberculin (PPD) was kindly supplied by the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey.

Immunizations

Crude F (5 mg/ml) or 250 μg/ml pure F was emulsified in Freund's complete adjuvant and 50 μl injected into the fore and hind foot-pads of mice.

Cell preparations

T cells. Lymph node cells were obtained from F-primed mice injected 10–20 days earlier in the footpads. Single-cell suspensions were enriched for T cells by passage over a nylon-wool column. Endogenous adherent cells were further depleted by incubating the nylon-wool non-adherent population on tissue culture petri dishes for 1 hr at 37°.

Spleen adherent cells (SAC). Pure F (200–500 μg), 2–5 mg crude F or 500–1000 μg PPD were injected in 0·3 ml intravenously into non-immune mice and the spleens removed from these animals 1 hr later. Control animals received 0·3 ml Hanks's balanced salt solution. Adherent cells were then prepared by plating the spleen cells on microexudate-coated petri dishes, as previously described (Sunshine, Katz & Feldmann, 1980). After 2 hr non-adherent cells were washed away and the adherent cells recovered by treating the plates with 3 mM ethylenediamine tetracetic acid (EDTA) for 15 min at 37°. These cells were then irradiated with 1000 rad from a ^{60}Co source.

Proliferation assay

Unfractionated lymph node or T cells (2×10^5) were mixed with graded numbers of irradiated SAC (usually $1–3 \times 10^4$) in 200 μl in flat bottom Microtitre plates (Falcon). All assays were set up in quadruplicate in RPMI 1640 containing 5% heat-inactivated foetal calf serum, 2 mm glutamine, 10 mM HEPES and 5×10^{-5} M 2-mercaptoethanol, in an atmosphere of

10% CO_2 at 37°. At 90 hr 1 μCi [^{125}I]UdR was added and 6 hr later the cells were disrupted using a cell harvester (Dynatech) and radioactive DNA was assessed by standard gamma counting. Results are expressed as mean ± standard deviation.

RESULTS

Usually the addition of soluble antigen in culture to a whole lymph node population prepared from animals which have been preimmunized in the footpads with that antigen results in high levels of T (and B) lymphocyte proliferation (Corradin, Etlinger & Chiller, 1977). We therefore tested first to see if T cells from high responder mice primed against allo F would respond to boosting with antigen *in vitro*. Figure 1 illustrates, however, that the effect of adding purified F to lymph node suspensions from F-primed AKR mice is inhibitory. Although as expected there is good proliferation to the adjuvant antigen, PPD, and no proliferation to an irrelevant antigen (chicken gamma globulin), adding purified F results in lower than background incorporation at 96 hr. This inhibitory effect is identical to the protein's effect on polyclonal mitogenic responses (Lane & Silver 1977) as well as being consistent with immunosuppressive effects of cruder liver preparations (Schumacher *et al.*, 1974). Concentrations down to 10 μg/ml F were tested and had an identical effect on lymph node suspensions from mice primed against crude or purified F. A lowering of proliferation was also seen when F was added to lymph node suspensions which had been passed over nylon-wool and tissue culture petri dishes (Fig. 1b) suggesting that F was acting directly on T cells.

Since it had been shown that antigen could be pulsed onto macrophages in the peritoneal cavity and in such a form was much more immunogenic than free antigen alone in adoptive transfer reactions (Mitchison 1969), we surmised that it might be feasible to pulse antigens onto splenic presenting cells by intravenous injection. Preliminary experiments indicated that a small but significant fraction of radiolabelled F injected intravenously rapidly associated (1–2 hr) with spleen cells

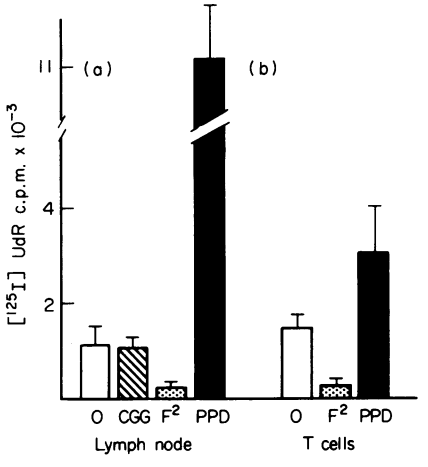

Figure 1. Free F inhibits the proliferation of F-primed lymph node cells. Draining lymph node cells prepared from AKR mice primed in the footpads with crude F^2 in Freund's complete adjuvant 10 days earlier. Single cell suspensions of whole lymph node or T cells incubated with antigens for 90 hr in quadruplicate microwell cultures. [^{125}I]UdR (1 μCi) added for further 6 hr. Results expressed as c.p.m. ± SD.

Cells (2×10^5) + no antigen (□); 100 μg/ml chicken gamma globulin (■); 100 μg/ml purified F^2 (▦); 50 μg/ml PPD (■).

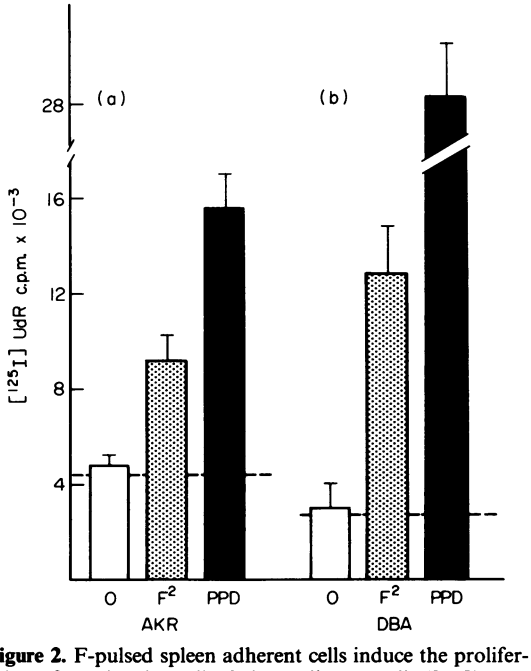

Figure 2. F-pulsed spleen adherent cells induce the proliferation of F-primed T cells. Spleen adherent cells (SAC) were prepared from non-immune animals injected i.v. 1 hr earlier with medium, crude F^2 or PPD. SAC irradiated with 1000 rad and coincubated for 96 hr with lymph node T cells from mice primed 14 days earlier with crude F^2 in Freund's complete adjuvant. [^{125}I]UdR 1 μCi present for final 6 hr. Results expressed as c.p.m. ± SD.

T cells (2×10^5) + 3×10^4 syngeneic SAC prepulsed with medium (□), crude F^2 (▦) or PPD (■). T cells with no SAC (– – –).

which adhered to microexudate-coated plates. We then tested to see if these *in vivo* pulsed cells could present antigen to primed T cells. Figure 2 indicates that T cells from AKR mice primed with allo F in Freund's complete adjuvant proliferate in response to spleen adherent cells which had been pulsed *in vivo* with either crude F or PPD, indicating that T-proliferative responses to F can be obtained when the antigen is presented in a cell-bound rather than free form.

However, Fig. 2 also indicates that proliferative responses can be obtained in mice which do not make an antibody response to F (DBA/2). One possible explanation is that as crude liver extract rather than pure F was used to pulse the presenting cells the T cells were responding to a non-F antigen in the extract.

It was therefore important to show that F-specific responses could be obtained. The ability to proliferate specifically to F^2 was shown by purifying the crude liver extract as described in the Materials and Methods. After elution from a carboxymethyl cellulose column two major protein peaks were obtained, only one of which reacted with anti-F antisera. This F^+ material was used to prime AKR mice. SAC were then pulsed *in vivo* with equivalent amounts (200 μg) of material from the F^+ and F^- peaks. Figure 3 illustrates that T cells from AKR mice primed with pure F^2 proliferate in response to the F^+ but *not* to the F^- material pulsed onto SAC. Interestingly these SAC can derive from syngeneic (AKR) or H-2 and F compatible mice (CBA).

The specificity of the response to this purified material was further investigated. Figure 4a shows that when T cells from DBA/2 mice primed with purified F^2 are coincubated with DBA/2 spleen cells pulsed with pure F^2 no proliferation is seen, parallelling the antibody response, and in contrast with the proliferation seen when crude extracts were used (Fig. 2). Under identical conditions T cells from AKR mice could respond to F^2 pulsed on to syngeneic SAC (Fig. 4b). Figure 4b also illustrates that T cells from F^2-primed AKR mice do not proliferate though in response to F^1 pulsed on to AKR SAC, demonstrating the specificity of the presenting cell T-cell interaction for the priming antigen.

All the foregoing data were obtained using allo F-primed T cells (F^2 into type F^1 mice). The ability to respond to syngeneic F was also tested. T cells from

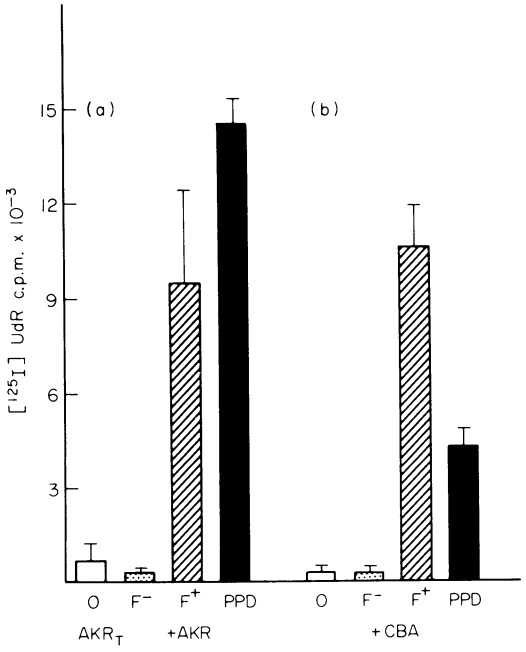

Figure 3. T cells from pure F^2-primed AKR respond to F^2-pulsed AKR and CBA SAC. AKR mice primed with Ouchterlony positive fraction (F^+). SAC pulsed with Ouchterlony positive (F^+ ▨) or negative (F^- ▤) fractions, PPD (■) or medium (□). Details as for Fig. 2. (a) 2×10^5T $\times 10^4$ AKR SAC; (b) 2×10^5T $+ 3 \times 10^4$ CBA SAC.

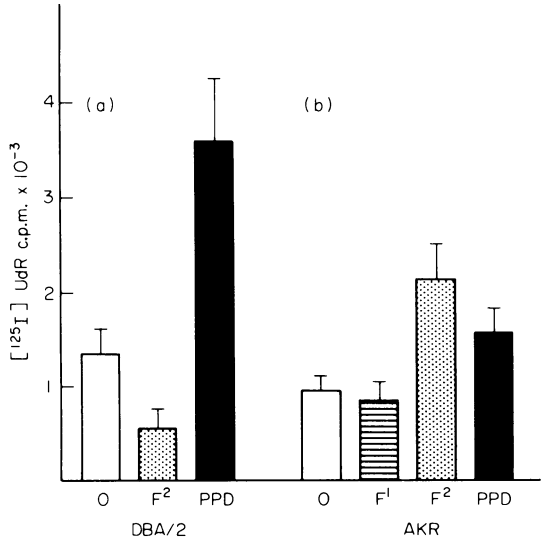

Figure 4. DBA/2 T cells do not respond to pure F^2-pulsed DBA/2 SAC. Legend as for Fig. 2. (a) 2×10^5 DBA/2 T cells $+ 3 \times 10^4$ DBA/2 SAC prepulsed with medium (□), pure F^2 (▤) or PPD (■); (b) 2×10^5 AKR T cells $+ 3 \times 10^4$ AKR SAC. Also prepulsed with pure F^1 (▤).

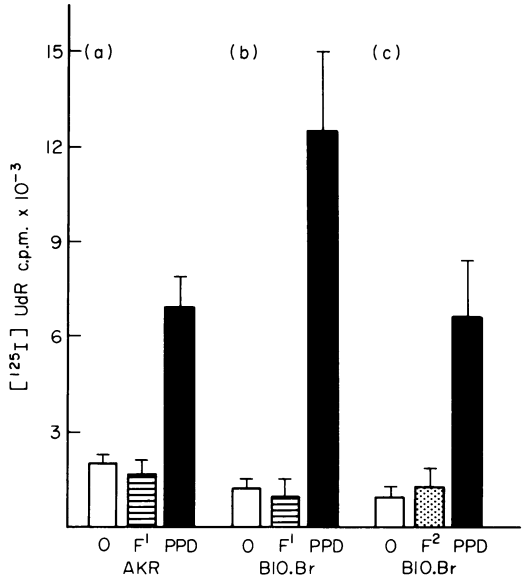

Figure 5. Mice do not respond to syngeneic F. See text for details. 2×10^5 T cells incubated with 3×10^4 syngeneic SAC prepulsed with medium (□), F^1 (■), F^2 (▨), or PPD (■). (a) F^1-primed AKR T cells. (b) F^1 primed B10.Br T cells. (c) F^2 primed B10.Br T cells.

AKR mice (type F^1) primed with purified F^1 were coincubated with F^1 pulsed AKR spleen cells. Figure 5a illustrates that there is no proliferation to F^1 antigen although the cells are able to respond to the adjuvant antigen. This would indicate that, as in the antibody response, syngeneic F cannot induce an immune response. However, we have not been thus far successful in showing even allogeneic responses to F^1 in this assay using B10.Br animals (type F^2), Fig. 5B. We have found, nonetheless, that T cells from syngeneically (F^2) primed B10.Br mice do not respond to F^2 pulsed syngeneic SAC (5c) using identical conditions to those used for demonstrating (allo) F^2 responses in AKR mice, supporting the above contention that syngeneic F does not induce T-cell or antibody responses.

DISCUSSION

In this present study we have established a system for investigating responses to the autoantigen F *in vitro*. The system makes use of the proliferative response of F-primed T cells to F-pulsed spleen adherent cells. Pulsing the antigen onto spleen adherent cells before exposure to T lymphocytes avoids the previously described problems of F-mediated inhibition (Lane & Silver, 1977). This technique may be of general use in studying immune responses to other potentially suppressive or lymphocytotoxic materials such as lectins or proteases.

The pattern of the *in vitro* proliferation data shows striking similarities to *in vivo* anti-F antibody production: (i) mice injected with pure syngeneic F do not show reactivity to F either *in vitro* (Fig. 5) or *in vivo* (Lane & Silver, 1976) and (ii) T cells from animals that do not make anti-F antibodies cannot respond to F *in vitro* (Fig. 4). It therefore appears that the *in vitro* proliferation system is a good analogue of the *in vivo* anti-F antibody response.

Possible explanations for the failure of T cells to respond to syngeneic (auto) F in this system are that the cells potentially able to respond have been either removed early in ontogeny (clonal deletion) or alternatively inactivated by regulator cells (suppression). [Failure to process and present autoantigen by antigen processing cells has not been formally excluded by these experiments but has been by other studies (Czitrom, Mitchison & Sunshine, 1981)]. Evidence for which of these mechanisms applies in the maintenance of tolerance to F is still inconclusive: chimaeric studies have been interpreted as supporting a clonal model but regulation by suppressor cells cannot be ruled out (Czitrom *et al.*, 1981). Clonal deletion has been invoked in responses to some autoantigens where no antigen-binding T cells have been detected (Clagett & Weigle, 1974) but on the other hand there is evidence to suggest that failure to respond may be due to a balance between autoreactive helper cells and a suppressor cell since under certain circumstances such as multiple priming antibody can be produced to a T-dependent autoantigen (EIRehewy, Kong, Giraldo & Rose 1981).

It is clearly important in measuring *in vitro* responses that purified F be used since the T-proliferation assay also appears to detect non-F antigens when crude liver extracts are used as a source of F. It is also striking that in the DTH assay for F non-F antigens may be recognized in crude liver extracts (Cooper, Coffey, Anders & Mackay, 1980). This common feature of the T-cell assays is in marked contrast to the *in vivo* antibody response where after immunization with liver extracts anti-F antibody production is dominant (Fravi & Lindenmann, 1968). A further correlation between the T-cell assays is also suggested by the fact that neither the delayed-type

hypersensitivity (DTH) nor proliferation system is responsive to F^1 as an alloantigen (Cooper *et al.*, 1980). It has been reported, however, that the elicitation of antibody responses to F^1 requires multiple boosting (e.g. Lane & Silver 1976) which is not used in the T-cell systems, suggesting that as currently designed both DTH and proliferative assays are below the threshold of detection of the weaker anti-F^1 responses.

These data lend support to the notion that antibody production and proliferation employ the same T-cell set. Both T-helper and proliferating cells have previously been shown to be Lyl^+23^- (Feldmann, Beverley, Dunkley & Kontiainen 1975; Schrier, Skidmore, Kurnick, Goldstine & Chiller, 1979) but it is possible that there are subtle differences in the composition of the T-cell subsets since T cells from mice that do not make antibody responses to synthetic antigens under Ir gene control, such as TGAL, can neither proliferate in response to TGAL (Schwartz & Paul, 1976), nor act as helpers for an anti-TGAL response (Howie, 1978) but can act as helpers for an anti-DNP response when the carrier is TGAL (Howie, Feldmann, Mozes & Maurer, 1977).

The nature of the antigen-presenting cell used in this assay has been described previously (Sunshine, Katz & Czitrom, 1981). The 2 hr splenic adherent cells used in this system are highly enriched for phagocytic FcR-bearing cells (75%–98%), 50% $I\text{-}A^+$ and radiation resistant. We therefore consider this population to be predominantly splenic macrophages. Dendritic cells may also be a potential comtaminant of this population. Since we have shown that purified dendritic cells can present antigen to primed T cells and purified macrophages cannot (Sunshine *et al.*, 1980), it is possible that all the presenting cell activity in our SAC population is attributable to dendritic cells, but this has not been shown after *in vivo* injection. It is clear, however, that a very small but significant fraction of injected antigen can become associated with the adherent population and is sufficient to trigger T-cell responses *in vitro*.

One further point concerning the antigen presenting cell population: earlier data suggested that in the absence of macrophages, antigen could induce tolerance (Dresser & Mitchison, 1968) and it has recently been shown that removal or depletion of antigen-presenting cells can lead to antigen being diverted along pathways of non-responsiveness rather than the activation of helper T cells (Toews, Bergstresser & Streilein, 1980). Since we have shown that free F inhibits the proliferation of T cells depleted of presenting cells it is likely that F may bind directly to T cells and that this may lead to an activation of suppressor cells when free F is added directly to primed lymphoid suspensions. By ensuring that allo F is processed by the presenting cell population before contact with T-cells activation rather than suppression of T-cell responses may ensue.

Finally, we have shown that T cells from F^2-primed AKR mice are responsive to F^2 pulsed onto SAC from CBA mice which are H-2 and F compatible and also make a strong anti-F antibody response. Since the F system is under the control of non H-2 genes (Silver & Lane, 1981) and F_1 crosses of F responder × non-responder strains can show dominant non-responsiveness in antibody production (Silver & Lane 1975), the way is now clear for exploring the influence of non-H-2 gene expression at the level of T cell–presenting cell interactions.

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REFERENCES

CLAGETT J.A. & WEIGLE W.O. (1974) Role of T and B lymphocytes in the termination of unresponsiveness to autologous thyroglobulin in mice. *J. exp. Med.* **139,** 643.

COOPER P.C., COFFEY M.F., ANDERS R.F. & MACKAY I.R. (1980) Delayed-type hypersensitivity to liver F antigen in the mouse. *J. Immunol.* **124,** 790.

CORRADIN G., ETLINGER H.M. & CHILLER J.M. (1977) Lymphocyte specificity to protein antigens 1. Characterization of the antigen induced *in vitro* T cell-dependent proliferative response with lymph node cells from primed mice. *J. Immunol.* **119,** 1048.

CZITROM A.A., MITCHISON N.A. & SUNSHINE G.H. (1981) I-J, F and hapten conjugates: canalisation by suppression. In: *Immunobiology of the Major Histocompatibility Complex, 7th International Convocation on Immunology* (Ed. C.J. Abeyounis, K. Kano and M.B. Zaleski), p. 243. Karger, Basel.

DRESSER D.W. & MITCHISON N.A. (1968) The mechanism of immunological paralysis. *Adv. Immunol.* **8,** 129.

ELREHEWY M., KONG Y.M., GIRALDO A.A. & ROSE N.R. (1981) Syngeneic thyroglobulin is immunogenic in good responder mice. *Eur. J. Immunol.* **11,** 146.

FELDMANN M., BEVERLEY P.C.L., DUNKLEY M. & KONTIAINEN S. (1975) Different Ly antigen phenotypes of *in vitro* induced helper and suppressor cells. *Nature (Lond.)*, **258,** 614.

FRAVI G. & LINDENMANN J. (1968) Induction by allogeneic extracts of liver specific precipitating auto antibodies in the mouse. *Nature (Lond.)*, **218,** 141.

HOWIE S.E.M. (1978) *Antibody responses under I region control.* PhD Thesis, University of London.

HOWIE S.E.M., FELDMANN M., MOZES E. & MAURER P.H. (1977) *In vitro* studies on H-2 linked irresponsiveness. 1. Normal helper cells to TGAL and GAT in low and non-responder mice. *Immunology*, **39,** 291.

IVERSON G.M. & LINDENMANN J. (1972) The role of a carrier determinant and T cells in the induction of liver specific auto antibodies in the mouse. *Europ. J Immunol.* **2,** 195.

LANE D.P. & SILVER D.M. (1976) Isolation of a murine liver-specific alloantigen, 'F' antigen and examination of its immunogenic properties by radioimmunoassay. *Europ. J Immunol.* **6,** 480.

LANE D.P. & SILVER D.M. (1977) Inhibition of lymphocyte proliferation by murine liver-specific 'F' antigen. *Transplantn. Proc.* **9,** 1063.

MITCHISON N.A. (1969) The immunogenic capacity of antigen taken up by peritoneal exudate cells. *Immunology*, **16,** 1.

SCHRIER R.D., SKIDMORE B.J., KURNICK J.T. GOLDSTINE S.N. & CHILLER J.M. (1979) Propagation of antigen-specific T-cell helper function *in vitro*. *J. Immunol.* **123,** 2525.

SCHUMACHER K., MAERKER-ALZER G. & WEHMER U. (1974) A lymphocyte inhibiting factor from normal human liver. *Nature (Lond.)*, **251,** 655.

SCHWARTZ R.H. & PAUL W.E. (1976) T lymphocyte enriched murine peritoneal exudate cells II. Genetic control of antigen-induced T lymphocyte proliferation. *J. exp. Med.* **143,** 529.

SILVER D.M. & LANE D.P. (1975) Dominant non-responsiveness in the induction of autoimmunity to liver specific F-antigen. *J. exp. Med.* **142,** 1455.

SILVER D.M. & LANE D.P. (1977) Identification of the gene locus controlling dominant nonresponsiveness to F-antigen. *Immunogenetics*, **4,** 295.

SILVER D.M. & LANE D.P. (1981) Polygenic control of the immune response to F antigens. *Immunogenetics*, **12,** 237.

SUNSHINE G.H., KATZ D.R. & CZITROM A.A. (1981) Heterogeneity of stimulator cells in the murine mixed leucocyte response. *Europ. J. Immunol.* (In press.)

SUNSHINE G.H., KATZ D.R. & FELDMANN M. (1980) Dendritic cells induce T cell proliferation to synthetic antigens under Ir gene control. *J. exp. Med.* **152,** 1817.

TOEWS G.B., BERGSTRESSER P.R. & STREILEIN J.W. (1980) Epidermal Langerhans cell density determines whether contract hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* **124,** 445.