J. Hyg., Camb. (1978), **80**, 259
Printed in Great Britain

Relationship between lymphocyte response to tetanus toxoid and age of lymphocyte donor

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SUMMARY

An investigation of the *in vitro* responsiveness of peripheral blood lymphocytes to tetanus toxoid was undertaken to determine the extent to which such reactivity might reflect *in vivo* reactions to tetanus toxoid immunization. It was found that lymphocyte transformation in the presence of tetanus toxoid, as measured by the uptake of tritiated thymidine, did not differ significantly for the group giving local or systemic reactions to tetanus toxoid and an immune control group. However, a striking inverse relation between lymphocyte reactivity to tetanus toxoid and age of the cell donors of both groups became clear. Such an age-related decline in the *in vitro* responsiveness of peripheral blood lymphocytes to a specific antigen has apparently not been reported previously in man.

INTRODUCTION

Reports of occasional reactors to first injections of tetanus toxoid (Levine, Ipsen & McComb, 1961; Kittler, Smith, Hefley & Cazort, 1966; Griffith, 1967; White *et al.* 1973) and the development of delayed type hypersensitivity reactions in the mixed reactions produced by intracutaneous injections of toxoid (Facktor, Bernstein & Fireman, 1972; White *et al.* 1973) prompted our investigation of the extent to which lymphocytes from reactors are transformed in culture by tetanus toxoid. We found that, although three of five severe reactors did show an increased response, the most important variable governing the *in vitro* reactivity to tetanus toxoid in both reactors and non-reactors was the age of the immune cell donor.

MATERIALS AND METHODS

Subjects investigated

The reactor group, which numbered 28, were employees of British Leyland UK Ltd at Cowley, Oxford, who developed reactions to tetanus toxoid in immunization procedures as described by White *et al.* (1973). Local reactions of excessive pain, redness or swelling were reported by all reactors, and three of the reactors also reported some degree of nausea and malaise. Only two of the group reacted

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to first injections of tetanus toxoid. In accordance with the criteria given by White *et al.* (1973), five of the reactions were classed as severe and the rest as moderate. The age range for the reactor group was 24 to 59 years, but the majority (23 of 28) were in the 30 to 39 and 40 to 49 decades. Sixteen laboratory personnel who received a course of tetanus immunization or booster doses within a year of sampling, without any adverse reaction, provided the control group. In this group the ages ranged from 21 to 67 years with the majority (10 of 16) in the 20 to 29 decade or 50 years of age and over. Despite the difference in the distribution of age within each group, the mean age of the reactor group did not differ significantly from that of the control group. None of the donors was taking any medication at the time of sampling or suffering from any known disease.

Lymphocyte response to tetanus toxoid

Lymphocytes were isolated by a 'Ficoll-Triosil' gradient from heparinized blood (Perper, Zee & Mickelson, 1968) after storage at room temperature for 24 h in an equal volume of S-MEM (Suspension) medium (Gibco Bio-Cult Diagnostics Ltd, Paisley, Scotland) with penicillin, streptomycin and kanamycin (10000 U/ml; 10000 μg/ml; 10 000 μg/ml respectively), and HEPES buffer for lymphocyte preservation (H. Festenstein, personal communication). Duplicate cultures of washed lymphocytes (10^6) were exposed to final concentrations of 10 μg/ml and 50 μg/ml of tetanus toxoid, and the degree of cellular proliferation estimated by liquid scintillation counting of incorporated tritiated thymidine. The conditions of culture and pulsing with tritiated thymidine were similar to those described for tetanus toxoid by Geha, Rosen & Merler (1973) except that antitoxin-free fetal calf serum was used exclusively as serum supplement to avoid stimulation of lymphocytes by antigen–antibody complexes (Bloch-Shtacher, Hirschhorn & Uhr, 1968; Möller, 1969). At the end of the pulsing period, 150 μl volumes of the cultures were processed on glass fibre disks by a modification of a communal washing procedure (Penhale, Farmer, MacCuish & Irvine, 1974) in which the disks were not removed to flasks but left on lead-weighted plastic pin-boards during successive transfer through dishes containing 5% trichloracetic acid, phosphate buffered saline (pH 7·3) and absolute ethanol. After drying, the disks were placed in scintillation vials containing 5 ml of scintillation fluid consisting of 4 g/l PPO* and 0·1 g/l dimethyl POPOP† (Koch-Light Laboratories Ltd), and the emissions were counted on a Tracerlab ICN Coru/matic 200 Scintillation Counter. Results were expressed as $\log_{10}$ of the difference between the average ct/min/ml for stimulated and unstimulated (control) cultures.

Lymphocyte response to phytohaemagglutinin (PHA)

Because cellular activity is a more sensitive indicator of cellular damage than is dye exclusion (Tennant, 1964), the response of lymphocytes to PHA (Wellcome lot No. K8805) was used to investigate possible detrimental effects of storage on lymphocyte activity. Preliminary experiments were conducted to establish the conditions giving optimal lymphocyte response to PHA.

* 2,5-diphenyloxazole. † 1,4-Di-(2-(4-methyl-5-phenyloxazolyl))-benzene.

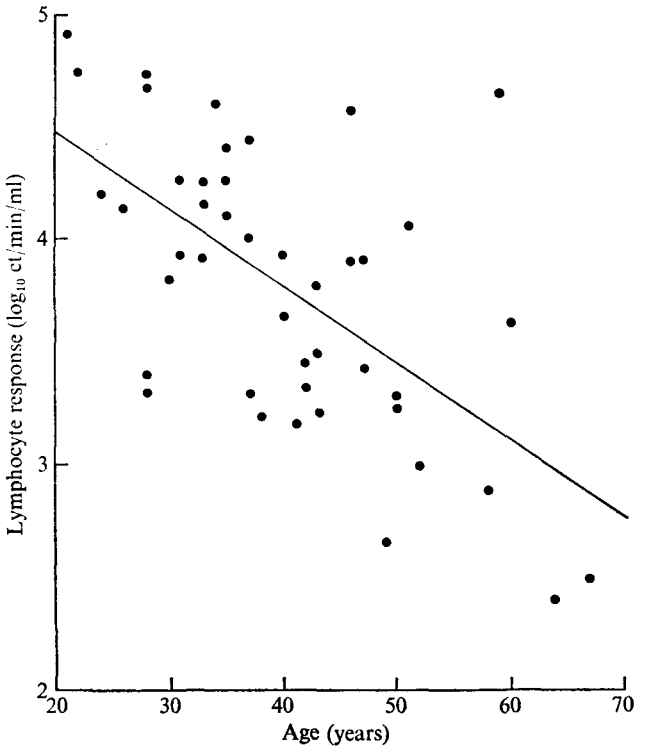

Fig. 1. Decreasing lymphocyte response to stimulation by 10 μg/ml of tetanus toxoid with increasing age for 43 subjects.

Tetanus antitoxin determinations

Tetanus antitoxin titrations were performed by P. A. Knight of the Wellcome Research Laboratories, Beckenham, Kent by a method based on that of Glenny & Stevens (1938).

RESULTS

Response to PHA

Maximum lymphocyte response to PHA was obtained when 10^6 lymphocytes were exposed to a final concentration of 1 μg/ml of PHA for three days with tritiated thymidine (1 μCi/ml) added 6 h before harvesting. When tested under these conditions and also at one-tenth optimal PHA concentration (Fitzgerald, 1971), the reactivity of lymphocytes preserved in S-MEM medium with antibiotics and HEPES buffer for 24 h did not differ significantly from that of lymphocytes processed without delay. Furthermore, in magnitude, consistency, and reproducibility, the lymphocyte responses to PHA resembled those reported by Fitzgerald (1972) and Yamamura (1973), thereby endorsing the reliability of the techniques used.

Response to tetanus toxoid

No significant differences were found in the lymphocyte response of the reactor and control groups at either concentration of tetanus toxoid when tested by

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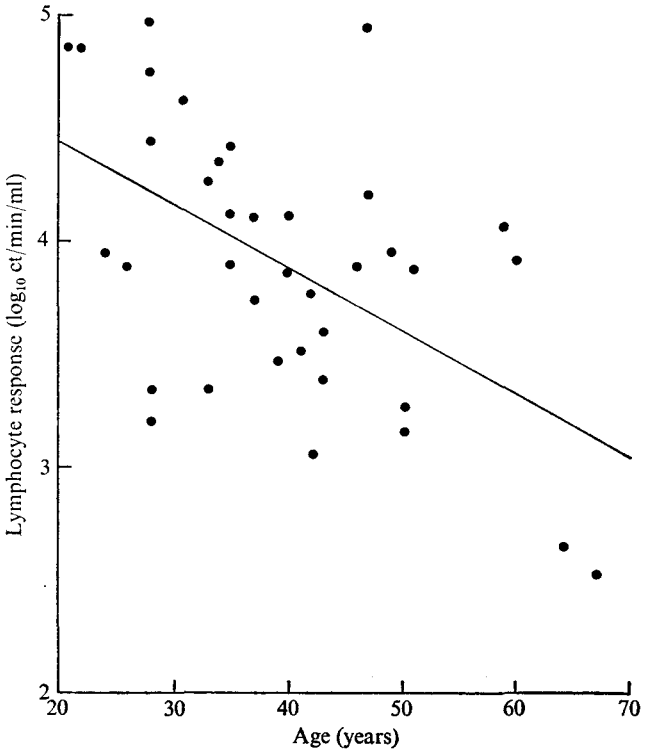

Fig. 2. Decreasing lymphocyte response to stimulation by 50 μg/ml of tetanus toxoid with increasing age for 37 subjects.

Student's t-test. Results for reactors and non-reactors were therefore combined to explore the effect of age. Figure 1 shows the lymphocyte response of 27 reactors and 16 non-reactors to 10 μg/ml of tetanus toxoid. Figure 2 shows the lymphocyte response of 21 reactors and 16 non-reactors to 50 μg/ml of tetanus toxoid. One of the subjects investigated did not respond to tetanus at a concentration of 10 μg/ml, and seven of them did not respond to 50 μg/ml of tetanus toxoid. Analysis of the response depicted in Fig. 1 gives a regression equation of $Y = 5{\cdot}1626 - 0{\cdot}0342x$, where Y is $\log_{10}$ ct/min/ml for thymidine uptake and x is age in years. The correlation coefficient (r) has a value of $-0{\cdot}607$ which is statistically highly significant ($P < 0{\cdot}001$). Similarly, the regression equation relating lymphocyte response to age of cell donor for 50 μg/ml of tetanus toxoid (Fig. 2) is $Y = 5{\cdot}0125 - 0{\cdot}0280x$, where Y is $\log_{10}$ ct/min/ml for thymidine uptake, and x is age in years. The value of r is $-0{\cdot}5287$ which is also highly significant ($P < 0{\cdot}001$).

The high responder in the upper age group in Fig. 1, with co-ordinates (x, y) of 59 and 4·65, developed extensive erythema and pain involving the whole of the upper part of the injected arm. The subject with co-ordinates 46 and 4·57 in the same figure was another reactor classed as severe. A large nodule was produced at the site of injection by the high responder with co-ordinates 47 and 4·95 in Fig. 2.

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Antitoxin determinations

The geometric mean antitoxin titres for reactors of moderate and severe degree were 0·97 and 0·91 AU/ml respectively, whereas that for the non-reactors was 0·52 AU/ml.

DISCUSSION

Unlike previous studies that have established reduced *in vitro* reactivity of lymphocytes to PHA with ageing of man (Pisciotta, Westring, de Prey & Walsh, 1967; Hallgren, Buckley, Gilbertsen & Yunis, 1973; Weksler & Hütteroth, 1973), our findings reveal an age-related decrease in lymphocyte response to a specific and widely-used antigen, tetanus toxoid.

In studies on the humoral immune response to tetanus toxoid, Tasman & Huygen (1962) found that average tetanus antitoxin titres were considerably higher in subjects less than 20 years of age than in those aged 20 years and older; and Newell *et al.* (1971) reported similar findings for women less than 25 years of age and those aged 25 years and older. Del Campo, Franzoni, Pasqualicchio & Tomasi (1970) showed that antitoxin titres determined 21 days after the second of two doses of tetanus toxoid given 28 days apart decreased progressively up to 90 years of age. Gottlieb *et al.* (1964) had earlier included a factor for age in their mathematical model for the evaluation of antitoxin titres following booster immunization.

The technique of lymphocyte transformation used in the studies reported here reflects the *in vitro* proliferative responsiveness of both T and B lymphocytes. That both cell types are involved was clearly demonstrated by Geha & Merler (1974) who showed that sensitized T cells, on exposure to tetanus toxoid, release a soluble lymphocyte mitogenic factor (LMF) which triggers a proliferative response in B cells in the presence of antigen. The LMF produced in response to tetanus toxoid is able to co-operate with more than one antigen in recruiting B cells to participate, thereby amplifying the initial response. It is this *in vitro* T-B cell co-operative response to tetanus toxoid that shows a steady decrease with age in immune subjects.

Three of the five severe reactors tested did show an enhanced lymphocyte response superimposed on the age-effect. Thus some correlation does exist between increased lymphocyte reactivity to tetanus toxoid and reactions to tetanus immunization, but it is restricted to severe reactions. The higher geometric mean antitoxin titres of the severe and moderate reactors, as compared with that for the non-reactor group, support the view that Arthus reactions probably account for much of the reactivity observed *in vivo* (Levine *et al.* 1961; Edsall *et al.* 1967; Griffith, 1967).

The influence of age on the *in vitro* response of lymphocytes to tetanus toxoid is apparent. Whether thymic failure in man is the primary event in the age-associated reduced response of lymphocytes to tetanus toxoid remains to be determined. However, support for the importance of the role of the thymus in the response of mice to tetanus toxoid has come from the studies of Hess, Cottier & Stoner (1963) who reported severely reduced secondary antitoxin responses to tetanus toxoid in thymectomized mice; and Willcox (1975) who showed that both the IgG and IgM

antibody response to tetanus toxoid are thymus-dependent in mice. Whatever the relative contribution of T and B lymphocytes to the age-related reduced responsiveness of man to tetanus toxoid, the early beginnings of the progressive decline is compatible with the concept that a significant deterioration of the immune system begins not with senescence but at the very prime of life, and may be causally related to the ageing process by predisposing towards diseases of ageing associated with immune dysfunction.

We are indebted to Professor H. Festenstein for his helpful guidance in the application of lymphocyte transformation techniques, especially in delayed testing. We thank Dr J. Nagel for supplying the tetanus toxoid, and Mrs R. Clifford for advice on statistics; Dr M. M. Peel was supported by a Research Training Fellowship from the Wellcome Trust.

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