Clin. exp. Immunol. (1978) 32, 459–465.

In vitro studies of suppressor cell function in human peripheral blood mononuclear cells

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(*Received* 16 *January* 1978)

SUMMARY

Peripheral blood mononuclear cells (PBMC) from normal donors, pre-cultured at 37°C for 24 hr before the addition of mitogen, demonstrated an enhanced proliferative response. This may be due to the loss of a subpopulation of suppressor cells during the incubation period.

Still further enhancement was observed when pre-culturing was prolonged for 48 hr, while cells pre-incubated at 4°C showed no increased responsiveness.

Concanavalin A (Con A) pre-activated PBMC suppressed the mitogen response of responder cells. More marked suppression was observed when the concentration of Con A used to induce the suppressor cells was increased. It was not possible to activate suppressor function in cells which had been kept *in vitro* for longer than 48 hr. These findings support the concept of the existence and function of suppressor cells, and that the suppressive influence is short-lived in *in vitro* culture.

INTRODUCTION

Regulation of the immune response is thought to be controlled by regulatory cells having suppressive or enhancing influences (Baker, 1975; Dutton, 1975). The net reactivity then represents the balance between these two forces (Gershon, 1974).

Suppressor cell function has been extensively investigated in rodents and their activity has been shown to be induced by a variety of immunological stimuli, including the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) (Dutton, 1972; Rich & Pierce, 1973; Folch & Waksman, 1974; Jacobs, 1975). Thus Dutton observed, using a plaque assay technique, that mouse spleen cells sensitized with sheep erythrocytes underwent a suppression of their antibody responses if Con A was added at the initiation of the culture. However, immune enhancement was seen when the addition of Con A was delayed for 24–48 hr. This enhanced response was interpreted as indicating the loss of suppressor cell function during the incubation period, prior to mitogen stimulation.

Reports on suppressor cell studies in man are few. Excessive suppression has been reported in variable hypogammaglobulinaemia (Waldman *et al.*, 1974), multiple myeloma (Broder *et al.*, 1975) and patients with fungal infections (Stobo *et al.*, 1976). Evidence for the absence of normal suppression in systemic lupus erythematosus (SLE) has been presented recently (Bresnihan & Jasin, 1977).

Con A induction of peripheral blood mononuclear cells (PBMC) which manifest suppressor function has now been demonstrated in man, in a manner analagous to that reported in rodent system (Shou, Schwartz & Good, 1976; Hubert, Delepesse & Govaerts, 1976; Bresnihan & Jasin, 1977). In these reports, Con A activation of PBMC has been shown to suppress the mitogen or antigen stimulation of a responder cell population.

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0099-9104/78/0600-0459$02.00

The concept of a short-lived suppressor cell in man, again comparable to that described in the mouse by Dutton (1972), was introduced by Bresnihan & Jasin (1977). Thus PBMC pre-cultured for 24 hr before mitogen addition showed significantly increased responsiveness when compared with cells stimulated at the commencement of culture.

The phenomenon of Con A-induced suppression and the enhanced stimulation of PBMC which follows the delayed addition of mitogen were both confirmed in this study. Other aspects of these two tests of suppressor cell function in man were investigated. The concept that suppressor cells *in vitro* are short-lived has been examined by allowing cells to age before attempting Con A-induced suppressive activity. Also, the possibility that an adaptive phenomenon might explain the enhanced responses which occur when the addition of mitogen is delayed was explored.

MATERIALS AND METHODS

Twenty-six healthy adults aged between 19 and 55 years were used as donors of normal PBMC.

Isolation of cells. Heparinized samples of blood were processed within 1·0 hr of collection. PBMC were obtained after plasma-gel (Laboratoire Roger Bellon) sedimentation and density gradient separation using Ficoll–Hypaque (Böyum, 1968). Cells were then washed in Hanks's balanced salt solution (HBSS) (Gibco, Biocult) supplemented with 10% heat-inactivated foetal calf serum and 50 u/ml of penicillin–streptomycin solution (Gibco, Biocult). Cells pre-treated with Con A and control cells were examined daily for 7 days to determine their viability, using the trypan blue dye exclusion test.

Mitogen preparation. Concanavalin A (Con A), Pharmacia, was dissolved in distilled water at a concentration of 1·0 mg/ml and then sterilized by filtration through a 0·22 μm Millipore filter (Millipore). Fresh dilutions of Con A were used for each experiment.

Loss of suppressor cell experiments. Cells were aliquoted to contain $0{\cdot}5\times 10^6$ cells per ml of culture medium. 0·2 ml volumes of medium containing 10^5 cells were added to the wells of a microculture plate (Sterilin microtitre F) covered with a sealer (Gibco, Biocult) and incubated at 37°C. All cultures were performed in triplicate and grown on their own (unstimulated) or in the presence of Con A (stimulated) at concentrations of 1·0, 2·0, 5·0 and 10 μg/ml. To one set of cultures the Con A was added at time 0 (t_0) and to another after cells had been cultured for 24 hr (t_{24}). In each instance the culture was terminated 48 hr after the addition of mitogen.

Activation of suppressor cell experiments. Aliquots of 4×10^6 cells were pre-cultured at 37°C in universal containers (Sterilin) at a concentration of 2×10^6 cells per ml. Con A (5·0 μg/ml) was added to one lot of cells but not to a control set. After a 48 hr incubation at 37°C, the cells were washed twice with HBSS and resuspended in RPMI containing 50 μg/ml mitomycin C (Sigma). After incubation at 37°C for 1 hr a further three washes in HBSS were performed. The respective Con A-treated and control cells were adjusted to counts of 10^6 cells per ml. Fresh autologous cells were prepared the same day containing 10^6 cells per ml.

To the microculture wells were added 0·1 ml of fresh autologous cells (responder cells) and an equal volume of mitomycin C-treated pre-cultured cells. This mixture of old and fresh cells was either left unstimulated or cultured in the presence of Con A. 10^5 cells were also cultured in the absence of old cells. All cultures were terminated after 48 hr.

Termination of cultures. After 44 hr incubation 0·3 μCi of [^{3}H]thymidine (sp. act. 2·0 mCi/mM; Radiochemicals) was added to each culture well. 4 hr later the contents of each well were harvested onto glass fibre discs (Whatman), washed in 10% cold TCA and dehydrated with methanol. Incorporation of radioactive thymidine was measured in a liquid scintillation counter (Tricarb, Packard) using a scintillation cocktail (Kent Koncentrate, Kent Laboratories, Canada).

Calculation of results. The base-line counts in the unstimulated cultures were subtracted from the counts in the stimulated cultures. In the loss of suppressor cells experiments, the data was analysed as in Bresnihan & Jasin (1977). The t_{24} counts per min (ct/min) were expressed as a ratio of the t_0 ct/min.

The information from the suppressor cell activation experiments was analysed as in Shou *et al.* (1976) in this manner:

$$1-\left[\frac{(Cm^{CA}-C^{CA})}{(Cm-C)}\right]\times 100\%;$$

where Cm^{CA} represents ct/min of normal lymphocytes plus Con A, with mitomycin C pre-treated cells cultured in the presence of Con A. C^{CA} is ct/min of normal lymphocytes plus Con A, with mitomycin C pre-treated cells; Cm is ct/min of normal lymphocytes plus mitomycin C pre-treated cells cultured in the presence of Con A; C is ct/min of normal lymphocytes plus mitomycin C pre-treated cells. This demonstrates the percentage inhibition or stimulation caused by the Con A pre-activated cells compared with control cultures.

Analysis of data. The Student's *t*-tests for paired and non-paired data were used to analyse the statistical significance of the results.

RESULTS

Delayed mitogen addition experiments

When PBMC were incubated at 37°C for 24 hr before the addition of Con A, a significantly increased response resulted at all three Con A concentrations, 1·0, 2·0 and 5·0 μg/ml ($P<0·001$). The ratios of enhancement of t_{24}/t_0 at 1·0 μg/ml Con A (4·9±0·05) and at 2·0 μg/ml (3·0±0·2) were significantly greater than the ratio when 5·0 μg/ml were used (1·9±0·1), with significant values $P<0·001$ and $P<0·01$ respectively (Fig. 1).

The effect of prolonging the pre-incubation period was investigated in two experiments (Fig. 2). Cells were pre-cultured for 24, 48 and 72 hr before mitogen stimulation. Even greater responsiveness was found when Con A was added to 48 hr old cells. However, cells pre-incubated for longer periods showed either no further enhancement or a diminution in response.

When PBMC were pre-incubated at 4°C instead of the customary 37°C, no enhanced mitogen response was noted (Fig. 3). However, if these cells were further incubated at 37°C for 24 hr, a marked increase in responsiveness resulted. This phenomenon was confirmed in two further experiments.

Activation of suppression experiments

PBMC pre-cultured with 5·0 μg/ml of Con A consistently suppressed the proliferation of fresh or 24 hr old responder cells, when compared with the effect of cells pre-cultured in the absence of Con A (Table 1). All the pre-cultured cells were also treated with mitomycin C and were shown in each instance to be unresponsive to mitogen stimulation. Thus, the only cells dividing in response to Con A in these experiments were responder cells. In control experiments to which no mitogen was added, these responder cells were neither stimulated nor inhibited by Con A pre-activated cells.

The percentage inhibition was most marked when the responder cells were stimulated with suboptimal concentrations of Con A (1·0 and 2·0 μg/ml), but significant inhibition was still observed when 10 μg/ml of Con A were used. The degree of inhibition of fresh cells was little different from that noted with 24 hr old responder cells.

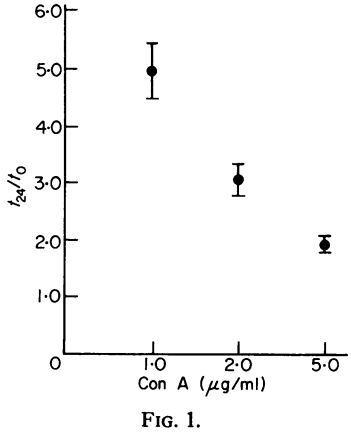

FIG. 1.

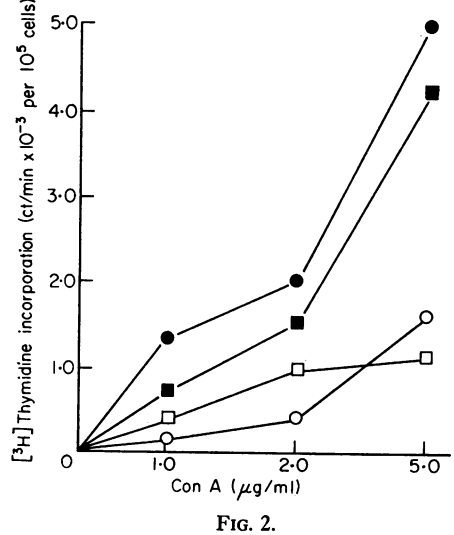

FIG. 2.

FIG. 1. Comparison of the effects of Con A on PBMC cultures stimulated after 24 hr (t_{24}) and on cultures stimulated at 0 hr. (t_0). Concentrations of Con A (1·0, 2·0 or 5·0 μg/ml) were added at t_0 or at t_{24} hrs. The relative increase of [³H]thymidine incorporation in cultures stimulated at 24 hr is represented by the mean ratio t_{24}/t_0 (±s.e.m.). The number of experiments carried out at 1·0, 2·0 and 5·0 μg of Con A were twenty-eight, thirty-six and forty-one respectively.

FIG. 2. The effect of prolonging the pre-incubation period of PBMC at 37°C for 0, 24, 48 and 72 hr before mitogen stimulation with various concentrations of Con A. (●) t_{48}, (■) t_{24}, (○) t_0 and (□) t_{72}.

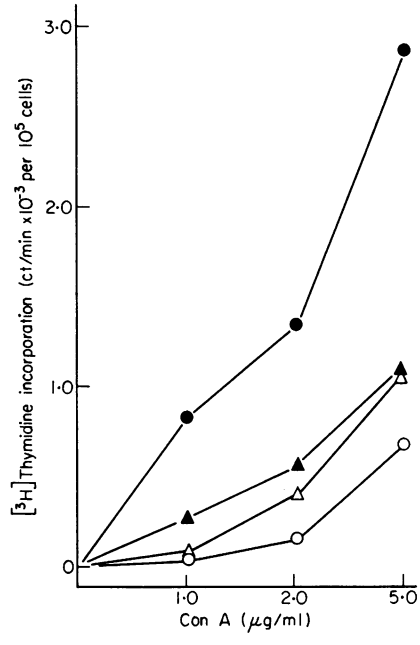

FIG. 3.

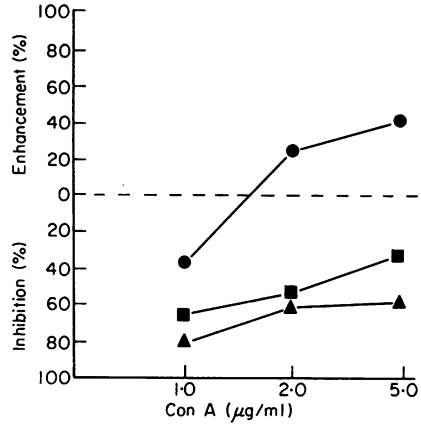

FIG. 4.

FIG. 3. A comparison of the effect of pre-incubating PBMC at 4°C for 24 hr before mitogen stimulation. Cells pre-incubated at 4°C for 24 hr (○), normal 2 day culture (△), cells pre-incubated at 37°C for 24 hr (▲), cells pre-incubated at 4°C for 24 hr and subsequently at 37°C for 24 hr (●). All cultures subsequent to pre-incubation periods were cultured for 48 hr in the presence of Con A (1·0, 2·0 and 5·0 μg/ml).

FIG. 4. PBMC were pre-activated with Con A (1, 5, 10 μg/ml) for 48 hr at 37°C and then treated with mitomycin C. The *in vitro* effect of 10^5 pre-activated cells on the response of 10^5 fresh autologous PBMC to various concentrations of Con A was then measured by [^{3}H]thymidine incorporation. The results were expressed as a percentage of enhancement or inhibition. (●) 2·0, (■) 5·0 and (▲) 10 μg/ml Con A.

Different concentrations of Con A were used to pre-activate PBMC in two experiments (Fig. 4). The suppression induced by 5·0 μg/ml was compared with the effect of 1·0 μg/ml and 10 μg/ml. The results indicate that 1·0 μg/ml Con A was capable only of inducing weak suppressive activity, whereas 10 μg/ml Con A caused even greater suppression than 5·0 μg/ml.

In a further series of experiments, cells were allowed to age before attempting the Con A activation of suppression (Fig. 5). Cells were incubated at 37°C for 24, 48, 72 and 96 hr before being activated with Con A. A general decrease in suppressor capacity was observed with age. Although 48 hr old cells could still be rendered suppressive, Con A pre-activation of cells beyond this time showed no suppression, but instead an enhancement effect.

Effects of mitomycin C-treated control cells on responder cells

Cells pre-cultured in the absence of Con A, and then mitomycin C-treated, had a considerable enhancing effect on 10^5 responder cells when subsequently cultured in the presence of this mitogen. This significant enhancement was found at all the Con A concentrations used, but was most marked at suboptimal doses (Table 1). At the concentrations of 1·0, 2·0 5·0 and 10 μg/ml of Con A, the significance values obtained were $P<0{\cdot}05$, $P<0{\cdot}0025$, $P<0{\cdot}025$ and $P<0{\cdot}05$ respectively.

DISCUSSION

The proliferative response to PBMC, as measured by [^{3}H]thymidine incorporation, was substantially improved by culturing these cells at 37°C for 24 hr before the addition of Con A. This effect was most

TABLE 1. Suppression of the mitogen responses of fresh and 24 hr old responder cells by the addition of pre-activated autologous lymphocytes at the different Con A concentrations

Con A (μg/ml)	Number of experiments	10^5 Responder* cells	Pre-activated† cells added	Control‡ cells added	Inhibition (%)	*P* value
1	5	450±257	264±96	904±388	61±7	< 0·01
2	9	684±307	494±127	1175±301	55±4·3	< 0·005
5	9	2092±415	2295±352	3405±644	34±2·5	< 0·0125
10	8	2411±345	2753±464	3806±657	25±7	< 0·05

The numbers in the table represent the amount of [^{3}H]thymidine incorporated by 10^5 responder cells (in ct/min).

* Represents PBMC cultured with Con A, 1·0, 2·0, 5·0 and 10 μg/ml, without the addition of pre-activated or control cells.

† PBMC were pre-activated by stimulation with Con A (5·0 μg/ml) for 48 hr at 37°C. The pre-activated cells were then treated with mitomycin C (50 μg/ml) for 1 hr at 37°C. 10^5 aliquots of these cells were then added to 10^5 responder cells and co-cultured in the presence of Con A (1·0, 2·0, 5·0 and 10 μg/ml) for 48 hr at 37°C.

‡ Control PBMC were treated in an identical fashion as the pre-activated cells, with the exception that they were pre-incubated in the absence of Con A, before the mitomycin C treatment and subsequent culture with 10^5 responder cells.

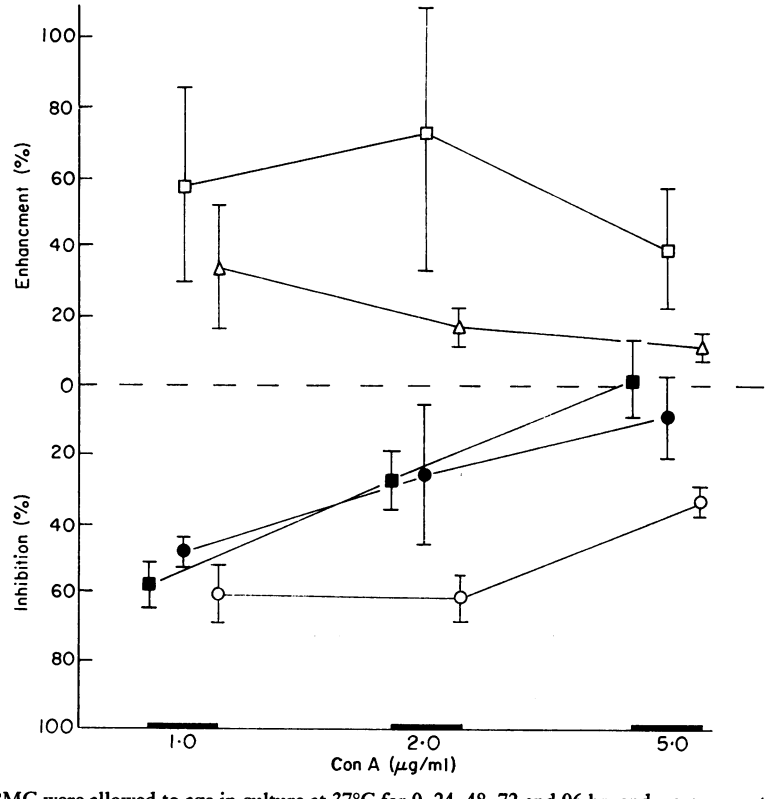

FIG. 5. PBMC were allowed to age in culture at 37°C for 0, 24, 48, 72 and 96 hr, and were pre-activated with Con A (50 μg/ml) for a further 48 hr at 37°C and then treated with mitomycin C. The *in vitro* effect of 10^5 pre-activated cells on the response of 10^5 fresh autologous PBMC to various concentrations of Con A was measured by [^{3}H]thymidine incorporation. The results were expressed as percentage enhancement or inhibition. The mean (± s.e.m.) of six determinations was plotted. (□) t_{72}, (△) t_{96}, (■) t_{24}, (●) t_{48} and (○) t_0.

marked when suboptimal concentrations of Con A were used. Nonetheless, responses were still significantly increased within the optimum range (these data are similar to those reported by Bresnihan & Jasin, 1977).

Still further enhancement was noted when cells were pre-incubated for a further 24 hr, or a total of 48 hr. Further prolongation of the pre-incubation period had a variable effect, with either no further enhancement or a diminution in response occurring. It would thus appear that PBMC in culture require up to 48 hr before the effect of their postulated short-lived suppressor cell population is lost.

It was proposed that this enhanced responsiveness was due to the loss of a subpopulation of suppressor cells that modulate the T-lymphocyte response. Evidence in favour of this concept was the finding that Con A-induced suppressor cells inhibited this enhancement (Bresnihan & Jasin, 1977).

The possibility that the increased responsiveness might be due to adaptation by the PBMC to the culture system was examined in this investigation. After such an adaptation, accelerated cellular proliferation might occur following mitogen stimulation. An analogous situation could be the lag phase in bacterial cultures, which is followed by rapid replication. Bresnihan & Jasin (1977) also considered adaptation as a possible explanation of the increased responsiveness, and tested it by washing the cells following the 24 hr incubation period and resuspending the cells in a fresh medium. However, the increased proliferation was not compromised by this procedure. To examine this hypothesis further, cells were incubated at 4°C instead of the normal 37°C. In this system short-lived cells should still die, but adaptation might be prevented. When cells were cultured under these conditions, no increased mitogen response was observed. When these cells were incubated at 37°C for a further 24 hr, enhancement then occurred. If the effect of suppressor cells in culture is short-lived, this result would suggest that more is involved than simple cell death. It is possible that these cells must undergo further change, which is prevented at 4°C, before becoming ineffectual mediators of suppression.

The mechanism by which mitogens stimulate lymphocytes to transform is thought to be related to the cross-linking of specific receptors on the cell surface (Greaves & Janossy, 1972; Allan & Crampton, 1973). Recent findings indicate that such receptors are mobile (Frye & Edidin, 1970) and therefore pre-incubation at 37°C before the addition of Con A could lead to membrane changes which are conducive to a greater stimulatory effect by Con A. Such membrane changes may be temperature-dependent, as is the case with capping by B lymphocytes, which occurs slowly at 4°C (Taylor *et al.*, 1971).

It is not likely that the enhancement phenomenon in these normal controls is due to the shedding of antibody directed against lymphocyte surface structures during the 24 hr pre-culture, as described in SLE by Wernet *et al.* (1974), since the spontaneous control cultures of t_0 and t_{24} cells showed no significant difference in amount of [^{3}H]thymidine incorporated.

PBMC pre-cultured with Con A suppressed the proliferation of fresh and 24 hr old responder cells. The findings presented here are similar to those previously reported (Shou *et al.*, 1976; Hubert *et al.*, 1976; Bresnihan & Jasin, 1977). This suppression was most effective when the responding cells were stimulated suboptimally. By increasing the concentration of Con A used to induce suppressor cells, more marked suppression was observed. However, at these higher concentrations suppression may be due to non-specific toxic effects, as suggested by Dutton (1972).

Since suppressor cells are reputed to be short-lived *in vitro*, experiments were conducted in which cells were allowed to age before attempting Con A-induced suppressor activity. The results showed some diminution of suppressor capacity in cells as they aged. Indeed, 72 and 96 hr old cells, in contrast, showed no suppressor capacity but had a stimulatory effect. This is in agreement with our observation in the delayed mitogen addition studies, that cells show a continuing enhanced proliferation up to 48 hr, but not beyond that age. Thus the potential anomalies of keeping cells *in vitro* for prolonged periods must be considered before a definitive interpretation of such findings is possible.

The treatment of activated suppressor cells with mitomycin C does not prevent their suppressor capacity. Therefore the replication of suppressor cells is not necessary for the exertion of their suppressive influence (Folch & Waksman, 1974). Control cells, cultured in the absence of Con A and then mitomycin-treated, have a significant enhancing effect on the responder cells. This is most marked when the responder cells are suboptimally stimulated, and this may be related to the reported release of

mitogenic factors from mitomycin-treated cells (Hirschberg, Rankin & Thorsby, 1974) or to an autologous MLC response. (Kuntz, Innes & Weksler, 1976). However, the uptake of [^{3}H]thymidine in the spontaneous control cultures of both Con A and mitomycin C pre-treated cells added to fresh cells, and mitomycin C pre-treated control cells added to fresh cells, showed no significant differences (P value $<0{\cdot}1$).

Thus the question may be asked whether the effect of Con A pre-activated cells is to inhibit non-specifically the enhancement caused by mitomycin-treated cells. This is not likely, however, because it has been demonstrated that even a small number of Con A pre-activated cells had a similar suppressive influence, although mitomycin was not used in the experimental procedure (Bresnihan & Jasin, 1977).

If Con A does indeed activate suppressor cells, which demonstrate their influence by the synthesis of suppressor proteins, an investigation of the effect of cycloheximide could provide confirmary evidence. This has been shown to inhibit suppressor cell function in the rat (Folch & Waksman, 1974).

We wish to thank Mrs Margaret Flynn for her secretarial help. This work was supported by the Medical Research Council of Ireland.

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