Clin. exp. Immunol. (1990) **82,** 429–434

Isotype-selective abrogation of established IgE responses

K. T. HAYGLASS & W. STEFURA *MRC Group for Allergy Research, Department of Immunology, University of Manitoba, Winnipeg, Canada*

(Accepted for publication 14 June 1990)

SUMMARY

Chemically modified allergens have been extensively studied in an attempt to develop materials of increased efficacy and improved safety for use in the immunotherapy of allergic disease. Most of the strategies that have been developed yield products that strongly inhibit *de novo* IgE responses but have only marginal impact on ongoing IgE responses. We report the virtual abrogation of pre-established murine anti-ovalbumin IgE responses using a glutaraldehyde-polymerized ovalbumin preparation (OA-POL) of M_r $3{\cdot}5 \times 10^7$. Secondary IgE responses are inhibited by 97–99% over a period of at least 8 months following three i.p. courses of OA-POL treatment. Administration of five additional ovalbumin [$Al(OH)_3$] booster immunizations over this period fails to alter this unresponsive state. The inhibition of antigen-specific IgE responses is isotype specific.

Keywords allergen immunotherapy IgE regulation modified allergens interferon-gamma

INTRODUCTION

Inhibition of ongoing *in vivo* IgE responses remains a major challenge. Various strategies that prevent very effectively the development of *de novo* IgE responses in animal models have proven to be much less successful in abrogating previously established IgE responses (reviews by Sehon, 1982; Ishizaka, 1985), leading some investigators to seek other approaches to controlling IgE responses.

In studies aimed at elucidating the control mechanisms that govern IgE production, we have utilized a murine model of human immediate hypersensitivity to ovalbumin. A high-molecular-weight soluble polymer of ovalbumin (termed OA-POL) which displays a highly restricted heterogeneity can be obtained by glutaraldehyde treatment of the native allergen following a procedure developed from those initially established in the 1970s (Patterson, Suszko & McIntire, 1973; Johanssen *et al.*, 1974). Treatment with 3–80 μg of this modified allergen prior to sensitization results in up to 99% inhibition of primary and secondary murine IgE responses. This ability to induce antigen-specific IgE-specific suppression is not genetically restricted by the MHC or Igh gene complexes (HayGlass & Stefura, 1990).

The capacity of modified allergens to regulate IgE responses has been examined most commonly in animal studies by treatment prior to sensitization. The capacity to abrogate ongoing IgE responses is much more difficult, but is considered to reflect more accurately the clinical challenge. We report here that treatment with OA-POL given following pre-sensitization of mice to ovalbumin leads to 97–99% inhibition of ongoing IgE responses. Moreover, this virtually unresponsive state is maintained over a period of more than 8 months despite five booster immunizations with ovalbumin in $Al(OH)_3$ adjuvant. The inhibition of anti-ovalbumin IgE responses induced by this class of chemically modified allergen is precisely targeted, displaying both antigenic and isotypic specificity.

Correspondence: Dr K. T. HayGlass, Department of Immunology, The University of Manitoba, 730 William Avenue, Winnipeg, Manitoba R3E 0W3, Canada.

MATERIALS AND METHODS

Animals

C57BL/6 mice (6–12 weeks old) and S-D rats were bred at the University of Manitoba breeding facility or were purchased from Charles River Canada (St Constant, Canada). All animals were maintained and used in strict accordance with the guidelines issued by the Canadian Council on Animal Care.

Preparation of chemically modified ovalbumin

Ovalbumin (grade VI, Sigma Chemical Co., St Louis, MO; or $\times 5$ recrystallized, ICN Biomedicals, Montreal, Canada) was dissolved at 25 mg/ml in sodium acetate/acetic acid buffer (0·1 M, pH 5·3), 0·5 pH units above its isoelectric point. Glutaraldehyde (Eastman Kodak, Rochester, NY) was added drop-wise with stirring over a period of several minutes to obtain a final molar ratio of 200:1 glutaraldehyde:ovalbumin. The reaction was allowed to proceed for 5 h to yield high molecular weight ovalbumin polymers. Following extensive dialysis against borate-buffered saline (0·1 M, pH 8·3), the solution was applied to a Biogel A-50m (BioRad Laboratories, Mississauga, Canada) gel filtration column ($2{\cdot}5 \times 90$ cm) for characterization and purification. The polymerized ovalbumin

was recovered as a single, sharp symmetric peak (Ve/Vo of 1·4 to 1·55), eluting at an average M_r of $3·5 \times 10^7$. This preparation, termed OA-POL, could be stored for at least 2 months at 4°C without evidence of change in its chemical or immunological properties. This method of glutaraldehyde modification was developed and used throughout this study in preference to one carried out at neutral pH in phosphate-buffered saline (0·1 M, pH 7·0) with all other reaction conditions as above, a procedure which yielded highly heterogeneous mixtures of reaction products (see Results).

Priming and treatment of mice

Mice were immunized by an i.p. injection of 0·2–2·0 μg ovalbumin or human serum albumin (HSA) (Sigma) adsorbed onto 2 mg $Al(OH)_3$ adjuvant in a total volume of 0·5 ml. A course of OA-POL treatment consisted of three 80-μg injections given in saline. Age-matched, saline-treated animals were used as controls, with naive 6–8-week-old mice included as additional controls in some long-term experiments. Blood was obtained by cardiac puncture 7–10 days after each immunization and the sera were stored at −20°C until analysed by ELISA or passive cutaneous anaphylaxis (PCA).

Determination of antigen-specific IgE

Anti-ovalbumin IgE levels were determined by 48-h PCA in female S-D rats as previously described (HayGlass & Strejan, 1984). Means of duplicate or triplicate analyses, rarely differing by more than one two-fold dilution in repeat assays, are presented.

Ovalbumin-specific ELISA

Specific antibody levels were determined in an alkaline-phosphate-based ELISA calibrated against a murine polyclonal anti-ovalbumin standard (1500 μg/ml IgG, kindly provided by Dr G. Strejan, University of Western Ontario, London, Canada). Briefly, ELISA plates (Corning 25805, Corning Science Products, Corning, NY) were coated overnight with ovalbumin at 200 μg/ml in carbonate buffer (0·05 M, pH 9·6), blocked with a 1% bovine serum albumin (BSA), 0·05% Tween 20 solution, and after extensive washing, serial dilutions of serum samples were incubated for 4 h at 37°C. The plates were then washed and an excess of alkaline-phosphatase-conjugated rabbit anti-mouse IgG, IgA or IgM (Southern Biotechnology Associates, Birmingham, AL) was added for incubation overnight at 4°C. After washing, *p*-nitrophenyl phosphate (Sigma) was added and the reaction was allowed to proceed for 30 min. Background control values from wells missing one component in turn did not exceed 0·07 absorbance units at 630 nm. Ovalbumin-specific IgG concentrations were calculated from the mean of at least three sample points falling within the linear portion of the titration curve of the internal standard which was run in each assay. For IgA and IgM, a standard of known anti-ovalbumin concentration in μg/ml was not available, so results were expressed as ELISA titres. These are expressed as the reciprocal serum dilution which gave an absorbance at 630 nm of 0·50 at 60 min. This absorbance was selected as an end-point as it was near the centre of the linear portion of the titration curves obtained in these assays. Results from duplicate or triplicate analyses were normalized against the constant anti-ovalbumin internal standard run in each assay. Variation between ELISA titres in different assays was usually less than 30% and always less than two-fold. Each serum sample was assayed at least twice. The isotypic specificity of each of the alkaline-phosphate-conjugated reagents was confirmed in separate assays prior to use.

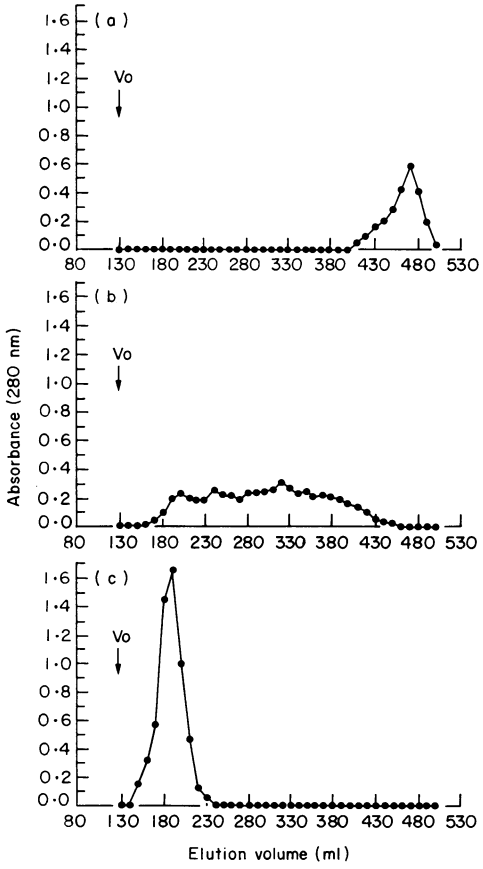

Fig. 1. Gel filtration profiles of ovalbumin; heterogeneous glutaraldehyde-polymerized ovalbumin; and glutaraldehyde-polymerized ovalbumin prepared at 0·5 pH units above the isoelectric point of ovalbumin (OA-POL). The relative heterogeneity of 25 mg of (a) unmodified ovalbumin; or heterogeneous glutaraldehyde-polymerized ovalbumin (b); or OA-POL (c) was evaluated by gel filtration on Biogel A-50m. Vo, void volume.

Statistical analysis

PCA and ELISA titres were log-transformed, following which geometric means were compared using unpaired two-tailed Student's *t*-test.

RESULTS

Characteristics of glutaraldehyde polymerized ovalbumin

Polymerization of protein allergens with di-functional cross-linking reagents such as glutaraldehyde usually leads to the generation of highly heterogeneous reaction products that vary widely in degree of modification and molecular weight (Patterson *et al.*, 1973, 1974; HayGlass & Strejan, 1984). Working with the model allergen ovalbumin, we previously defined conditions under which high molecular weight polymers of restricted heterogeneity could be prepared. Thus, although glutaraldehyde treatment at neutral pH yields a variety of polymers that range from dimers to very high molecular weight species (Fig. 1),

Table 1. Characteristics of glutaraldehyde-polymerized ovalbumin (OA-POL)

Effect on anti-ovalbumin response*	Primary response	Secondary response
IgE	16–64-fold decrease	7–32-fold decrease
IgG	10–40-fold increase	0–3-fold increase
IgA	No significant change	
IgM	No significant change	

† Relative molecular mass $3{\cdot}5 \times 10^7$

‡ Antigenicity 95% to >99% reduced

* Assessed by competitive inhibition radioimmunoassay and passive cutaneous anaphylaxis.

† Assessed by molecular sieve chromatography.

‡ As determined by immunization with ovalbumin in $Al(OH)_3$ 10 days after OA-POL pretreatment (3×80 μg intraperitoneally) in 14 inbred and one outbred murine strains.

chemical modification carried out 0·5 pH units above the isoelectric point of the model allergen used yields high molecular weight polymers of greatly restricted heterogeneity. These freely soluble molecules are not aggregates but represent polymers of approximate calculated molecular formula $ovalbumin_{800}$-to-$ovalbumin_{900}$ and are termed OA-POL. Very little if any ovalbumin remains unpolymerized under these reaction conditions as indicated by the absence of detectable protein at the elution volume observed for unmodified ovalbumin (Fig. 1). Table 1 summarizes the salient biological characteristics of OA-POL: major decreases in antigenicity, enhanced capacity to inhibit the induction of allergen specific murine IgE responses and maintained capacity to induce increased allergen specific IgG responses relative to unmodified ovalbumin.

Pretreatment with glutaraldehyde-polymerized ovalbumin

Choosing as our model the high responder C57BL/6 mice, the impact of OA-POL pretreatment on the development of primary and secondary antibody responses was examined. As can be seen in Fig. 2, treatment with three i.p. injections of OA-POL (80 μg) prior to ovalbumin [$Al(OH)_3$] immunization resulted in ≥98% suppression of IgE responses, major increases in primary IgG responses and no significant changes in IgA and IgM responses to ovalbumin. All injections of modified allergen were given intraperitoneally rather than intravenously, a route commonly used to elicit tolerance, in order to approximate more closely the clinical situation.

The effects were antigen specific, with antibody responses to unrelated antigens unaffected by OA-POL pretreatment. Thus, the capacity of C57BL/6 mice to mount *de novo* HSA-specific primary or secondary IgE responses was not affected by treatment with OA-POL (Table 2).

Abrogation of ongoing IgE responses

The capacity of OA-POL to inhibit previously established anti-ovalbumin IgE responses was evaluated in the same strain by using one to three courses of treatment (three 80-μg i.p. injections each) following ovalbumin [$Al(OH)_3$] priming. The data in Fig. 3, the mean of five independent experiments, demonstrate that ongoing IgE responses were markedly suppressed (98%, $P<0{\cdot}001$) by three courses of OA-POL treatment. IgG and IgM anti-ovalbumin responses in these mice were increased by two- and four-fold, respectively, while IgA responses to ovalbumin were unaffected ($P>0{\cdot}05$). One or two courses of treatment, although sometimes effective, failed to inhibit ongoing IgE responses suggesting consistently, that the effectiveness of treatment is proportional to the number of courses of treatment administered in this system.

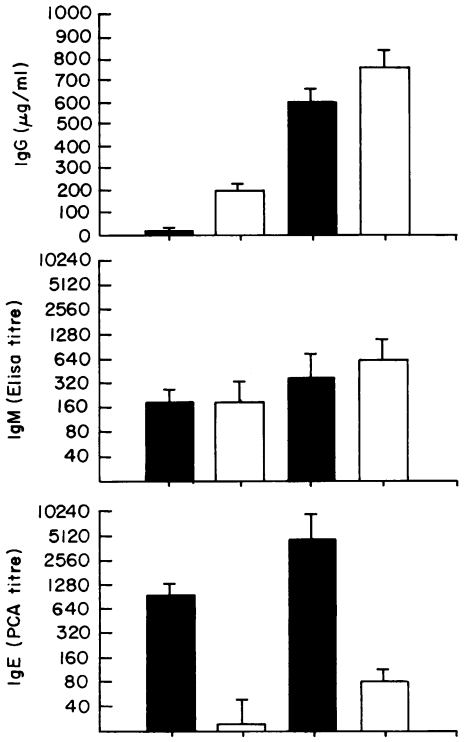

Fig. 2. The effect of pretreatment with glutaraldehyde-polymerized ovalbumin prepared at 0·5 pH units above the isoelectric point of ovalbumin (OA-POL) on the response of C57BL/6 mice to ovalbumin immunization. Saline or OA-POL (80 μg in saline) were injected intraperitoneally 10, 12 and 14 days prior to immunization with 2 μg ovalbumin in $Al(OH)_3$. The primary response was determined 10 days later. All mice were rechallanged with ovalbumin [$Al(OH)_3$] on day 28 and bled on day 35. Anti-ovalbumin IgE (PCA titres), IgG (μg/ml) and IgM (ELISA titres) are presented from one experiment representative of seven performed. Comparison using Student's *t*-test (unpaired) yields significant differences between untreated and OA-POL-treated groups for primary ($P<0{\cdot}001$) and secondary IgE responses ($P<0{\cdot}001$) as well as primary IgG responses ($P<0{\cdot}001$). ■, pretreated with saline, challenged with ovalbumin, primary response; □, pretreated with OA-POL, challenged with ovalbumin, secondary response.

The resilience of IgE selective unresponsiveness in mice that had been primed with ovalbumin [$Al(OH)_3$] and treated with OA-POL was examined by repeatedly immunizing with ovalbumin in $Al(OH)_3$ at approximately monthly intervals. The data in Table 3 demonstrate that the inhibition of ongoing IgE synthesis which was induced by OA-POL treatment is highly persistent (≥253 days) and resilient to further exposures to allergen (at least five booster immunizations) despite no further treatment with modified ovalbumin being administered. It should be noted that not only were IgE responses in the OA-POL-treated mice reduced to 1–3% of those observed in untreated controls, but

Table 2. Treatment with glutaraldehyde-polymerized ovalbumin (OA-POL) prior to immunization with ovalbumin leads to antigen-specific inhibition of IgE responses

		Anti-ovalbumin IgE response		Anti-HSA IgE response	
Treatment	Immunization	Primary	Secondary	Primary	Secondary
Saline	Ovalbumin	800	3200	—	—
OA-POL	Ovalbumin	40	160	—	—
Saline	HSA	—	—	100	320
OA-POL	HSA	—	—	80	400

Mice were treated with 80 μg OA-POL or saline 14, 12 and 10 days prior to immunization with 2 μg ovalbumin in $Al(OH)_3$ or HSA in $Al(OH)_3$. Mice were bled 10–14 days later, boosted on day 28 and bled on day 35. Peak primary and secondary responses are presented.

HSA, human serum albumin.

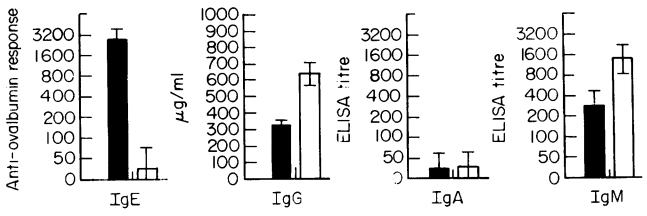

Fig. 3. Abrogation of established IgE responses upon treatment with OA-POL. C57BL/6 mice were primed with 0·2 μg ovalbumin in $Al(OH)_3$ on day 0. Beginning 2 weeks later, mice were treated with three courses of saline or OA-POL (3 × 80 μg, i.p.). All groups were rechallenged with 0·2 μg ovalbumin [$Al(OH)_3$] 10 days after completing OA-POL or saline treatment. Mean anti-ovalbumin IgE (PCA titre), IgG (μg/ml), IgA (ELISA titre) and IgM (ELISA titre) responses obtained 7 to 10 days later are presented. Student's *t*-test results: IgE, $P < 0{\cdot}001$; IgG, $P < 0{\cdot}05$; IgA, $P > 0{\cdot}05$; IgM, $P < 0{\cdot}05$. ■, immunized and rechallenged with ovalbumin, treated with saline; □, immunized and rechallenged with ovalbumin, treated with OA-POL.

more importantly, the OA-POL-treated mice failed to generate anamnestic IgE responses following rechallenge with the sensitizing allergen.

Antigenic specificity of IgE selective abrogation

To determine whether the abrogation of IgE responsiveness that resulted following OA-POL treatment of pre-sensitized mice was antigen-specific, C57BL/6 mice were primed with ovalbumin [$Al(OH)_3$] as previously, treated with three courses of OA-POL or saline, and challenged with ovalbumin or an unrelated antigen in $Al(OH)_3$ adjuvant. Alternatively, mice were immunized HSA, given OA-POL treatment and rechallenged with HSA. Neither the *de novo* development of IgE responses nor the recall of pre-established responses to this non-cross-reactive antigen was affected by OA-POL treatment (Table 4).

DISCUSSION

Over the past 15 years, a variety of different experimental approaches have been assessed in attempts to improve the safety of hyposensitization treatment for immunotherapy. Although ongoing anti-hapten IgE responses (e.g. anti-benzylpenicilloyl) have proven amenable to abrogation (Wetterwald *et al.*, 1986), established IgE responses to protein allergens have proven to be much less so (Sehon, 1982; Ishizaka, 1989). Here we report the capacity of a class of glutaraldehyde-polymerized protein allergens to inhibit strongly (97–99%) ongoing murine IgE responses. The effects of this treatment are both antigen specific and isotype specific.

Immunotherapy of allergic disease is one of the most frequently administered treatments in allergology and in the USA it is considered the first choice for adolescents (Bousquet & Michel, 1989). Good evidence exists that the process is effective only when used in appropriately selected patients with certain well-defined allergies (Norman, 1988; Ewan, 1989).

Unlike pharmacologic approaches, immunotherapy offers the potential of establishing changes in the underlying disease state. However, the occurrence of local and systemic anaphylactic reactions in some cases leading to fatalities, has resulted in restrictions being imposed in some countries (Committee on Safety Medicines, 1986) and a formal re-evaluation of the status of hyposensitization treatment by the World Health Organization (WHO) and the International Union of Immunological Societies (Thompson *et al.*, 1989). Central to its recommendations are the need for clinical hyposensitization therapy to develop approaches that offer increased safety and efficacy.

In the present report we selected an approach modelled after chemically modified allergens initially developed over a decade ago, assessed in clinical trials (Hendrix *et al.*, 1980; Grammer *et al.*, 1983) and related to those now in clinical use in Western Europe and Canada.

Our major objective in studies of allergens for immunotherapy lies not in potential direct clinical application, but in their value as experimental models allowing us to delineate the molecular mechanisms involved in the control of hypersensitivity. As some earlier studies indicated a relation between increased molecular mass and efficacy (HayGlass & Strejan, 1984) while others revealed that non-immunogenic low molecular weight proteins can be converted to IgE inducing immunogens upon glutaraldehyde treatment (Attallah, Kuroume & Sehon, 1975), we developed a modification of the original polymerization process to yield soluble, high molecular weight ovalbumin polymers of restricted heterogeneity. Treatment with these molecules led to highly efficient abrogation of established IgE responses in C57BL/6 mice.

Studies *in vitro*, and more recently *in vivo*, have indicated a central role for interleukin-4 (IL-4) and interleukin-5 (IL-5) in the generation of human and murine IgE responses (Pène *et al.*, 1988; Finkleman *et al.*, 1988b, 1990). There is good evidence that interferon-gamma (IFN-γ) acts as an antagonist to this process (Coffman & Carty, 1986; Snapper & Paul, 1987). Both direct administration of recombinant IFN-γ (rIFN-γ) and induction of IFN-γ synthesis (via *Brucella abortus* immunization) have been shown to reduce substantially IgE levels *in vivo* (Finkelman *et al.*, 1988a). Recent findings from mice treated with OA-POL prior to ovalbumin immunization indicate preferential induction of IFN-γ-secreting, ovalbumin-specific

Table 3. Persistence of IgE selective abrogation

Immunization (day 0)	Treatment (days 15, 30, 45)	Rechallenge (day 55)	Isotype	Anti-OA response day 63	day 98	day 127	day 156	day 205	day 253
Ovalbumin	Saline	Ovalbumin	IgE	16000	8000	4000	3200	5000	4000
			IgG	390	657	835	615	405	560
			IgM	420	1968	2400	2280	1632	1787
Ovalbumin	OA-POL	Ovalbumin	IgE	40	80	100	50	100	200
			IgG	935	501	833	645	430	649
			IgM	1872	1488	1512	1680	1020	1549

Mice were primed with ovalbumin $Al(OH)_3$ primed then treated with three courses of glutaraldehyde-polymerized ovalbumin (OA-POL) or saline and rechallenged with ovalbumin $Al(OH)_3$ as described for Fig. 3. At approximately monthly intervals thereafter (days 90, 120, 149, 198, and 245), mice were boosted with ovalbumin in $Al(OH)_3$, bled 7–10 days later and anti-ovalbumin IgE (PCA titre), IgG (μg/ml) and IgM (ELISA titre) levels were determined for the days indicated.

The data are from one of five experiments.

Table 4. Abrogation of ongoing IgE responses: antigenic specificity

Immunization	Treatment	Immunization	Peak IgE response Anti-ovalbumin	Anti-HSA
Ovalbumin	None	Ovalbumin	2560	< 10
Ovalbumin	OA-POL	Ovalbumin	80	< 10
HSA	None	HSA	< 10	320
HSA	OA-POL	HSA	< 10	320
Ovalbumin	None	HSA	< 10	200
Ovalbumin	OA-POL	HSA	10	160

Mice were immunized with ovalbumin (0·2 μg in $Al(OH)_3$) or HSA (2 μg in $Al(OH)_3$ as indicated above on day 0 and day 56.

OA-POL treatment consisted of three standard courses of three 80 μg i.p. injections in saline on alternate days beginning on day 15, day 30 and day 45. Peak ovalbumin-specific and HSA-specific IgE responses were measured on days 63–69.

HSA, human serum albumin; OA-POL, glutaraldehyde-polymerized ovalbumin.

CD4 T cells by this class of modified allergen but not by unmodified ovalbumin (HayGlass & Stefura, 1990). This raises the possibility that a similar mechanism may be responsible for the abrogation of ongoing IgE responses observed in the present report. Studies to resolve this issue are currently underway.

Taken as a whole, these data indicate that treatment with appropriately modified allergens may be useful for inhibition of well-established and *de novo* IgE responses *in vivo* and as an approach to further our understanding of IgE regulation. As might be expected, shutting off established IgE responses is more difficult than preventing their development. In the present system three times as many treatments were required to consistently achieve the same degree of inhibition of IgE responsiveness as was obtained in pretreatment experiments with one course.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Council of Canada. We thank Professor A. Sehon for critical discussions and his ongoing support and encouragement, Dr G. Strejan for providing useful reagents and Ms G. Falkenberg for secretarial assistance.

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