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Induction Of Antigen-specific, Isotype-selective Suppression Of Immunoglobulin-e Responses With Glutaraldehyde - Polymerized Ovalbumin

Kent Taylor Hayglass

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INDUCTION OF ANTIGEN-SPECIFIC, ISOTYPE-SELECTIVE SUPPRESSION OF IgE RESPONSES WITH GLUTARALDEHYDE-POLYMERIZED OVALBUMIN

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ABSTRACT

Polymerization of protein allergens with glutaraldehyde (GA) has been shown to lead to decreased local or systemic adverse reactions when used in human hyposensitization therapy. At the same time, clinical trials have revealed that their immunotherapeutic value was at least as good, and in many cases significantly better than that of the corresponding conventional native allergen extracts. Despite these advantages their mechanism of action remains unclear.

The purpose of this study was to determine the characteristics and classification of immunosuoppressive activity established as a consequence of treatment with GA-proteins.

Treatment of mice, a widely accepted animal prototype for human allergy, with GA-polymerized ovalbumin (OA-POL) led to highly efficient antigen-specific suppression of primary and secondary IgE responses. On the other hand, IgG antibody and delayed hypersensitivity responses were not suppressed. Decreased binding to antibodies against the native OA molecule occurred with all GA-modified OA preparations regardless of whether they were polymerized or not. On the other hand, the ability to suppress IgE responses appeared to correlate directly with the size of the polymers used. Suppression was transferable to syngeneic recipients and depended upon the presence in the donor spleens of live Thy 1.2+ and Lyt 1+ lymphocytes.

OA-POL treatment of mice previously immunized with OA (alum) abrogated ongoing IgE responses. Not only were IgE anti-OA responses 8-10 fold lower in the treated group, but anamnestic IgE responses were prevented. Abrogation persisted for over 300 days despite multiple OA

(alum) boosters. These results, taken in combination with experiments demonstrating undiminished antigen- and IgE isotype-specific suppression more than seven months after a single course of OA-POL pretreatment, suggested the existence of memory suppressor T cells. Direct evidence for such cells was obtained in adoptive transfer experiments where memory Ts cells were shown to be long-lived ($\geq$ 200 d), rapidly recallable and displaying greater suppressive activity than conventional "primary" suppressor cells.

The study suggests that homeostasis of IgE responses may be maintained, at least in part, by long-lived and boosterable antigen-specific, isotype-selective suppressor T cells. The experimental manipulation of such cells indicates that increasing their activity may provide a useful approach for the selective regulation of IgE- mediated hypersensitivity.

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ABBREVIATIONS

BBS	borate buffered saline
BSA	bovine serum albumin
BSS	Hanks' balanced salts solution
C	complement
CFA	complete Freunds adjuvant
DNP	dinitrophenol-hapten
DTH	delayed hypersensitivity
GA	glutaraldehyde
KLH	keyhole limpet hemocyanin
MHC	major histocompatibility complex
OA	ovalbumin (unmodified)
OA_1-L	lightly modified, unpolymerized ovalbumin
OA_1-H	heavily modified, unpolymerized ovalbumin
OA_4, OA_{175}, OA-POL	ovalbumin polymers, see Table I
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
PRW	glutaraldehyde polymerized ragweed allergens
RIA	radioimmunoassay
Th	helper T lymphocyte
Ts	suppressor T lymphocyte
Tsm	memory suppressor T lymphocyte

INTRODUCTION

The discovery of IgE as a distinct class of skin-sensitizing antibodies by Ishizaka et al (1966) and its immunochemical characterization by Bennich (Johansson & Bennich, 1967) can be considered to mark the beginning of modern research into immediate hypersensitivity. Since that time, considerable progress has been made towards a clearer understanding of how IgE synthesis occurs, how its production is regulated and the means by which it initiates the series of events associated with immediate hypersensitivity.

Delineation of the central mechanisms regulating IgE responses may provide valuable clues for the understanding of regulatory interactions governing immune responses as a whole (Katz, 1980). Such study has already provided broad insights into immunoglobulin gene rearrangement (Yaoita et al., 1982), mechanisms of immune tolerance (Ishizaka, 1980), and immunodeficiency diseases (Buckley et al., 1978) At the same time, this research may provide us with new and more effective tools for the management of IgE mediated allergic disease in humans.

Establishment of animal prototypes capable of boosterable IgE antibody responses provided a model for human atopic disease (Revoltella & Ovary; 1969, Levine & Vaz, 1970). The necessity of selecting appropriate high responder strains of mice, adjuvant, schedules and routes of immunization for persistent IgE production was established in the early 1970's and has been extensively reviewed (Ovary, 1981; Lehrer & Bozelka, 1982).

Unsuccessful attempts to stimulate IgE production in nude mice (Michael & Bernstein, 1973) and in neonatally thymectomized rats

(Okumura & Tada, 1971) soon established the strong T dependence of IgE responses. It was later shown that although B_ϵ cells can be primed (proliferation without differentiation) with both T dependent and T independent antigens in nude mice, the participation of helper T cells (Th) is essential for the formation of IgE-producing plasma cells or of long-lived B memory cells (Okudaira et al., 1980)

Collaborative interactions between B and Th cells of a type similar to those previously established for most antibody production in other Ig classes (Mitchison, 1969), was shown by Ishizaka (Okudaira & Ishizaka, 1973) to be required for murine IgE production as well. thus, recognition of carrier determinants by Th cells leads ultimately to production of anti-hapten antibody by B_ϵ cells which differentiate into IgE producing plasma cells or B_ϵ memory cells. However, the functional similarities between B_ϵ-Th interactions and B_γ-Th interactions do not necessarily mean that the same helper T cell cooperates with both B cells. Studies in both rabbits and mice during the mid 1970's produced evidence supportive of discrete, isotype-selective helper T cell populations (Kishimoto & Ishizaka, 1973 a; Kishimoto & Ishizaka, 1973 b; Hamaoka et al., 1974; Kojima & Ovary, 1975).

During this same period, cells capable of suppressing IgE antibody responses were first observed. Rats, characteristically displaying very low, unboostable IgE titers, could be converted to patterns of sustained Ab synthesis of considerable magnitude by experimental manipulations such as low dose irradiation or cyclophosphamide treatment (Tada, 1978). The finding that such enhanced IgE responses could be terminated by transfer of syngeneic

thymocytes or spleen cells provided direct evidence that the limited, transitory IgE levels in normal rats were a consequence of a dominant suppressor T cell (Ts) mechanism. A variety of experiments (Reviewed in Katz, 1980) demonstrated that similar manipulations would selectively increase the magnitude of IgE production in mice.

Concurrently it was found that 3-5% of normal unprimed murine splenocytes are surface IgE^+ in spite of very low serum IgE concentrations and that an identical number of B_ϵ were present in high, low and non-responder mice (Teale et al., 1981).

Taken as a whole, this information suggested that differences in IgE responsiveness were not attributable to simple variations in the quantity of B cells available for antigenic stimulation, nor that faulty macrophage processing was responsible (as is often the case for non-responders in other Ig classes), but rather that the regulation of IgE synthesis is normally dominated by suppressive mechanisms that limit IgE production. The realization that natural (immunopathological) or experimental decreases in Ts cell numbers or activity led to enhanced IgE production suggested to several investigators that increases in Ts activity might have the opposite effect, thus providing an effective means of limiting IgE responses.

Experimental Strategies for the manipulation of IgE responses

(i) Tolerance

Considerable effort has focused on identifying those stages of IgE induction that are most amenable to suppression. One approach is direct tolerization of mature B_ϵ cells. Antigen- and IgE isotype-selective tolerance has been induced in both naive and

previously sensitized animals by several methods. Repeated feeding of protein antigens (Vaz et al., 1977), injection of antigen covalently coupled to nonimmunogenic carriers such as isologous gamma globulin (Filion et al, 1980) synthetic polyaminoacid copolymers such as D-GL (Katz & Borel, 1978) or polysaccharides (Watanabe et al, 1977), and a variety of other protocols have successfully established B cell tolerance.

Alternatively, because the differentiation of B_ϵ cells to IgE forming plasma cells is known to be highly T dependent, one might suppress antibody formation by reducing helper T cell activity. This was attempted by the repeated intravenous injection of deaggregated ovalbumin. Long-lived Th cell tolerance was successfully induced in the absence of demonstrable suppressor T cells (Colby & Strejan, 1980).

(ii) Idiotype Suppression

The occurrence of identical or cross-reactive idiotypes among antibodies and lymphocyte receptors of different individuals within the same inbred strain has made it possible to regulate certain immune responses by the corresponding anti-idiotypic antibodies (a-id). This approach may prove particularly fruitful when the IgE response is of restricted heterogeneity.

The effects of passively administered isologous a-id antibodies on IgE and IgG anti-benzylpenicilloyl hapten (a-BPO) responses in BALB/c mice were studied. Following immunization with BPO-carrier conjugates and treatment with a-id, these mice displayed depressed anti-BPO levels for 2-3 weeks without change in their response to the

carrier protein (Blaser et al., 1980). Induction of autologous a-id responses was achieved in naive or BPO-primed mice by immunization with affinity purified anti-BPO antibodies obtained from separate syngeneic BPO immunized mice. These mice, actively producing anti-id antibodies, exhibited long-term (≥25 weeks) suppression of anti-BPO IgE and IgG responses when boosted with BPO-carrier conjugates (Blaser & de Weck, 1982).

Analogous experiments gave similar results for the induction of unresponsiveness to the hapten phosphorylcholine, a major antigenic determinant of Diplococcus pneumoniae (Blaser et al., 1979). Anti-hapten anti-id antibodies are now known to induce hapten-specific Ts cells which selectively suppress the formation of the relevant Id with anti-hapten specificity.

Suppression of IgE responses to protein antigens, of particular interest here was also achieved following the induction of autologous a-id. Thus, anti-OA antibodies produced in different individuals of the same strain were used to immunize BALB/c mice and resulted in the production of anti-"anti-OA idiotype". IgE and IgG responses to haptens presented for immunization as hapten-OA conjugates and, to a lesser extent, anti-OA responses themselves were suppressed. The exact mechanism of IgE antibody suppression by anti-id against anti-carrier antibodies remains unclear (Blaser et al., 1981). Two important caveats to the concept of therapeutic idiotype suppression should be noted. Induction of auto-anti-id in humans may be significantly harder to achieve. Even should such induction be possible, idiotypic shift might constitute a problem. Secondly, passive administration of a-id to humans has been shown to elicit

Prausnitz-Kustner reactions, presumably by crosslinking of mast cell-bound id+ IgE. Active induction of anti-id antibodies might result in recurrent allergic phenomena such as chronic urticaria. Whether the concentration of anti-id induced by treatment would be sufficiently high to cause such negative side effects remains to be seen.

(iii) T suppressor cells: Isotype-specific

Perhaps the most intensive area of study in IgE immunoregulation consists of those strategies designed to enhance Ts numbers/activity. The most prominent consequence to date is that no consensus can be reached on the nature of Ts cell(s). Excellent, often contradictory, evidence supports the existence of IgE class-specific Ts cells, antigen-specific Ts cells, and concomitantly antigen- and class-specific Ts cells.

Initial identification of antigen non-specific Ts cells in low responder SJL mice was immediately followed by reports that these cells were IgE class-specific. Elimination of Lyt 1, but not of Lyt 2, cells abolished the capacity of SJL spleen cells to suppress IgE responses (Watanabe et al., 1976; 1977). Suppressive factors later isolated from these cells were shown to be free of Ig determinants and greater than 300,000 d. molecular weight.

DNP-coupled mycobacteria have been used to elicit class-selective Ts cells in BALB/c mice. Suppression was hapten-specific for its induction but non-specific in its effect as IgE responses to a variety of non-crossreactive antigens were also suppressed. (Reviewed in Kishimoto, 1982). Recent experiments by the same group with

phosphorylcholine-mycobacterium conjugates support their previous findings (Kishimoto et al., 1981).

Also supportive of antigen-non-specific regulatory mechanisms selectively acting upon murine IgE responses are the data obtained by Katz and colleagues. An essential role for mycobacterium is indicated for this system as well. IgE selective Ts cell activity was enhanced in mice injected with complete Freund's adjuvant. The serum of such mice was found to contain two soluble factors, one enhancing (EFA), and one suppressive (SFA), that affect IgE responses in an isotype-specific manner (Katz, 1980). These factors could be obtained from both low and high responder mice and acted across MHC and species restriction barriers (Katz et al, 1980). SFA:EFA ratios are thought to determine the magnitude of IgE responses.

Thus, several investigators argue that features of IgE responses not easily demonstrated in IgM or IgG antibody responses could be explained by the existence of IgE class-selective helper and suppressor T cells and their soluble factors. Kishimoto (1982) suggests that if natural in vivo IgE responses are really regulated by a balance of $T_{H\epsilon}$ and $T_{S\epsilon}$ cells, it is conceivable that induction of an increased number of IgE class-specific Ts cells might result in selective diminution of IgE production while sparing that of other Ig isotypes.

(iv) T suppressor cells: Antigen-specific

Antigen-specific Ts cell activity that is class non-selective in its expression has been elicited in murine models by several approaches. Of particular interest here are those strategies

utilizing chemically modified protein antigens.

Beginning in 1975 Ishizaka et al. carried out an extensive study of the chemical and biological characteristics of urea-denatured protein allergens. Denatured, aggregated Antigen E and OA derivatives were prepared by the use of 8 M urea. Although losing the capacity to combine with antibody, these preparations were found to be capable of priming antigen-specific mouse Th cells if they were injected in association with adjuvant (Ishizaka et al., 1975; Takatsu & Ishizaka, 1975). In contrast, intravenous injection of urea-denatured-ovalbumin (UD-OA) into OA-primed mice suppressed both IgE and IgG antibody responses. Carrier-specific Ts cells were demonstrated by adoptive transfer (Takatsu & Ishizaka, 1976). Consequent in vitro studies demonstrated that 24 hour incubation of splenic lymphocytes from UD-OA treated mice with OA or OA-bearing macrophages enhanced their suppressive activity (Takatsu & Ishizaka, 1977).

Sehon has prepared a variety of conjugates of allergen with non-immunogenic molecules in the belief that antigen in non-immunogenic form favors selective activation of Ts cells (Lee & Sehon, 1978; Sehon & Lee, 1981). Treatment of mice with antigen-polyvinyl alcohol (PVA) or antigen-polyethylene glycol (PEG) conjugates resulted in antigen-specific suppression of IgE and, to a lesser extent, IgM and IgG responses. Later studies involved grafting monofunctional PEG (mPEG) onto protein allergens to obtain substituted monomers rather than the heterogeneous copolymers obtained with the polyvalent PEG's initially used. mPEG-antigen conjugates were also immunosuppressive due to the generation of antigen-specific Ts cells.

(iv) Antigen-and Isotype-Specific Suppression

Intravenous treatment of IgE producing mice with milligram amounts of highly substituted acetoacetyl-OA conjugates has been shown to markedly reduce the animals' ability to respond to one subsequent immunization with OA (Bach & Brashler, 1975). No concomitant suppression of hemagglutinating or total serum antibody was noted. Significant differences between treated and control animals were not found upon later challenge. The mechanism of suppression was not examined in this system.

Pretreatment of mice with OA-pullulan (a linear polymer of glucose) also resulted in excellent antigen- and isotype-specific suppression of IgE responses (Usui & Matsuhashi, 1979). Although suppression was demonstrated following adoptive transfer, its T cell dependence was not determined; nor were the effects of treatment upon previously established IgE responses examined.

More recently, saturated fatty acids (C_{12}, C_{14}, C_{16}, C_{18}) were chemically coupled to OA and these hydrophobically modified antigen conjugates were used to treat BALB/c mice (Segawa et al., 1981). IgE responses of both naive and primed mice were suppressed in an isotype- and antigen-specific manner when palmitic acid-OA conjugates were used. However, the medium to long-term efficiency of such treatment is unknown as good anamnestic IgE responses were evident in treated groups and only a single OA (alum) booster was administered to test the resilience of suppression. Ts cells were demonstrated by adoptive transfer but because the levels of IgG in the recipients were not reported, the isotypic specificity of these Ts cells remains unclear.

Studies of Human Allergy

From the beginning of this century a concerted attempt has been made to determine the genetic factors underlying human susceptibility to atopy. Considerable progress has been made towards understanding hyperresponsiveness of the immune system as a whole as well as the genetic basis for immune responsiveness to particular allergens. Single gene hypotheses, previously accepted as explanatory of hypersensitivity, have been supplanted by the realization that allergy is multifactorially determined (Marsh et al., 1980; Bonini et al., 1983). Although both genetic and environmental factors (i.e. allergen exposure, presence and duration of breast feeding) have been strongly implicated in determinng sensitivity, studies of identical twins reared apart indicate that genetic influences upon total Ig and specific antibody levels far surpass the influence of different environmental backgrounds (Kohler et al. 1982).

It has been suggested that both HLA Ir genes as well as genes outside the MHC are responsible for determining IgE levels (Black & Marsh, 1978). Predisposition towards hypersensitivity is currently believed to be regulated by multiple genes inherited as Mendelian recessive traits. Attempts to recognize linkage disequilibria, associations of two or more loci more commonly in atopics than if totally random assortment is made, have met with rather limited success to date.

Therapeutic intervention in allergy can take many forms. The pharmacological approach is of major importance. A wide variety of agents, many highly effective, have been developed for symptomatic relief. It must be conceded however that this approach is at best

palliative and does not result in any change in the underlying state of hypersensitivity.

Immunologic approaches have been extensively studied in both basic science and clinical settings. The purpose is to interfere immunologically at any of several stages leading to the production of elevated levels of antigen-specific IgE in the sensitive individual. Implicit is the assumption that decreased serum IgE correlates with decreased sensitization of mast cells/basophils, hence lower sensitivity to the allergen. It is now generally accepted that a concentration-dependant correlation exists between serum IgE and the in vivo number of IgE molecules per basophil.

Immunologic intervention for the treatment of allergy is conventionally dated from the studies of Noon (1911). Development of an effective hyposensitization regime and accurate assessment of its efficacy has often been hampered by poor experimental design, small samples and insufficient controls (Reviewed in Norman, 1982). Currently, conventional hyposensitization procedures, established through empirical clinical experience, usually involve a large number of injections of minute doses of the sensitizing allergen. Best results are obtained when there is only one offending allergen, in younger patients, and when higher doses of extracts can be tolerated. Treatment must be considered potentially dangerous as maintenance doses are usually considerably larger than those capable of inducing anaphylactic shock (Overell, 1978). Indeed, a high degree of local and general reactions are not uncommon and, although not usually serious, constitute a very real drawback of therapy. Although treatment is usually discontinued (after a couple of years) if marked

improvement is not noted, in at least one report patients have been continued on conventional immunotherapy for 35 years before another approach was tried (Small and Shapiro, 1981).

One of the first well controlled, double blind trials of hyposensitization was conducted in 1954 (Frankland & Augustin, 1954), 200 individuals with grass pollen fever, asthmas or both were treated with a mixture of grass pollen extracts (unmodified), an ultrafiltrate or burnt sugar placebos. 79% of hayfever and 94% of allergic asthma patients reported good to excellent results. Improvement was noted in 32% of the placebo treated subjects. A large number of studies since that time have established that after a temporary increase in IgE levels, most patients receiving immunotherapy tend to display a blunting or absence of post-seasonal IgE rises and ultimately, decreases in IgE production asociated with symptomatic improvement as measured by symptom score indices.

Considerable research has been directed towards relating clinical improvement to quantifiable immunologic parameters. In addition to identifying a correlation between decreased antigen-specific serum IgE and symptom score indices, decreased basophil sensitivity to ragweed antigen E has often been shown to correlate with symptomatic improvement (Norman, 1982). Increases in allergen-specific IgG are commonly found in patients undergoing hyposensitization, many of whom improve. It should be understood however that many exceptions have been reported so changes in immunological parameters are of no firm predictive value (Lichtenstein et al., 1971). In fact correlation between clinical relief and immunological findings has proven difficult in a wide range of studies (Norman, 1982).

Immunologic Mechanisms of Hyposensitization

The actual mechanism of hyposensitization is an even more contentious issue. In 1935 Cooke (Cooke et al., 1935) showed that some human sera would bind allergens when incubated with pollen extract in vitro and so block the chain reaction when sensitive individuals were exposed to that particular sample. These "blocking" antibodies, which could be distinguished from IgE or reagins, were initially demonstrated in patients who had received desensitization injections. Later, blocking antibodies were recognized in the serum of both treated and untreated atopics and were shown to be allergen-specific IgG (Lichtenstein et al., 1968). They were presumed to block the allergic response in vivo by combining with allergen in the fluid phase, preventing its interaction with cell-bound IgE. Many hyposensitization programs are expressly designed with the intention of elevating IgG and this, rather than changes in IgE, is often the parameter measured.

Although a significant correlation has often been found between increases in IgG and decreases in IgE following treatment (Yunginger & Gleich, 1973; Gleich et al, 1977), many studies have failed to find such a correlation (Lichtenstein et al, 1968; 1973; Chapman et al, 1980; Wolfe, 1982).

One school of thought holds that hyperproduction of IgE is causally related to decreased Ts cell function (Buckley & Becker, 1978; Strannegard & Strannegard, 1981). The emphasis in therapy thus shifts from increasing blocking antibody levels to increasing suppressor cell function. Reduced numbers of IgG Fc receptor positive T cells, (Tγ), a subset believed to include suppressor T cells

(Moretta et al, 1977), have been observed in peripheral blood of patients with respiratory allergic diseases. Following specific hyposensitization which resulted in a sharp improvement in clinical symptoms, the T_γ subset regained normal values (Canonica et al., 1979; Rivlin et al., 1981; Jensen et al., 1981). More recently the availability of monoclonal antisera recognizing helper (OKT4) and suppressor/cytotoxic (OKT 8) cells has allowed confirmation of a quantitative and functional suppressor cell deficit in children with atopic eczema (Butler et al., 1982). Suppressive effects of OKT 8 T cells upon IgE responses are currently under investigation in several systems (Ohta et al, 1983; Cooper et al, 1983).

Whether decreased Ts levels are of primary pathogenic importance to the development of allergy or are secondary to the disease itself is not firmly established. However, prospective studies done on one month old infants who later developed atopic disease support the notion of a primary defect in these children (Juto & Strannegard, 1979).

It has been suggested that future modes of immunotherapy may lie in the development of regimens which increase Ts numbers (Hughes et al., 1982). Indeed the improvement in human antigen-specific Ts activity noted following successful hyposensitization for ragweed allergy (Rocklin et al., 1980) suggests that this parameter can be manipulated. Preliminary reports from several laboratories support the contention that the activity of Human IgE-suppressor cells can be manipulated (Nagaya, 1982; Hassner & Saxon, 1983).

Chemically Modified Allergens In Human Immunotherapy

One approach increasing patient safety and shortening the tedious

and expensive injection schedules associated with conventional immunotherapy is the use of chemically modified allergens. The characteristics of an optimal modified antigen were initially defined as decreased ability to crosslink mast cell-bound IgE and retained capacity to induce IgG (Overell, 1978). Any alterations in the structure of the allergen molecule should not result in the introduction of major new antigenic specificities that could themselves lead to induction of sensitivities. The modified preparation should of course be as effective for treatment as native, unmodified allergen.

Preliminary results of human clinical trails using several modified antigens initially studied in animal prototypes are now available. Use of Urea-denatured AgE for almost two years resulted in suppression of seasonal rises in IgE upon natural reexposure to ragweed pollen (Norman et al., 1980). There was however, no evidence of suppression of ongoing IgE synthesis nor obvious improvement in clinical symptoms. Treatment with methoxy PEG_{5000}-substituted AgE also provided scant evidence of suppression other than a blunting of the usual postseasonal IgE rise. In a separate study treatment with D-glutamic acid: D lysine substituted AgE, methoxy PEG_{2000}-AgE or lauryloxy PEG_{1200}-AgE gave only slight evidence of decreased IgE near the end of a 6 month trial (Norman et al., unpublished data).

A unique approach to decrease allergenicity for treatment purposes utilizes formaldehyde under mild reaction conditions to form allergoids in a manner analogous to that employed for the conversion of toxins to toxoids (Marsh et al., 1969, 1981). A six year clinical study with ragweed allergoids demonstrated a slow but consistent

decline in serum IgE and a statistically significant decrease in patient symptoms (Norman et al., 1981; 1982). Immunotherapy of grass pollinosis with allergoids is also effective (Georgitis et al., 1983).

Glutaraldehyde Modified Protein Antigens

Treatment of proteins with GA leads to polymerization via covalent crosslinking of ϵ-amino groups of amino acid side chains. Although the actual reaction sequence leading to polymerization remains unclear, it has been suggested that crosslinkages of the 4° pyridinium type are formed (Hardy et al., 1976; 1979). The extent of polymerization and size of polymers generated depends upon a number of conditions including relative concentration of protein and GA, pHt and reaction time (Strejan & Surlan, 1979).

In the belief that GA-modified allergens might provide a worthwhile tool for the management of atopy, Patterson, polymerized ragweed AgE. The heterogeneous population of polymers obtained was fractionated by gel filtration into preparations of $2x10^5$-$4x10^6$d. and 4-$20x10^6$d. Both polymer preparations were capable of inducing IgG in guinea pigs and rabbits at least as well as monomeric AgE (Patterson et al., 1973). In further studies, polymerized ragweed antigens (PRW), formed from a crude aqueous extract of ragweed pollen, displayed 100-1000 fold decreased capacity to react with human IgE from sensitive individuals as measured by Prausnitz-Kustner tests and basophil release assays (Patterson & Suszko, 1974).

The relationship between GA modification and immunogenicity was further investigated by Moran and Wheeler (Moran & Wheeler, 1976; Wheeler et al., 1976). GA polymerization of Timothy grass pollen

extracts gave protein products that retained their immunogenicity for IgG production in rats and rabbits. Rats treated with such polymers displayed a decreased capacity to bind or induce allergen-specific IgE

Attempts to correlate molecular weights of such polymers with immunological properties have been hampered by the high degree of heterogeneity in the preparations studied. Use of small polymers has resulted in enhancement of IgG production while treatment with highly aggregated preparations resulted in a reduction in allergen specific IgG relative to treatment with unmodified protein. Some preparations lost the ability to stimulate IgE antibody production as a result of GA modification (Patterson & Suszko, 1974; Wheeler et al., 1976). Others were shown to aequire immunogenicity when under similar conditions of immunization the native molecule was non-immunogenic (Attallah et al., 1975). The efficiency of GA-protein polymers of different sizes in inducing IgE suppression has not been examined.

The major finding of these early studies (decreased allergenicity following GA-modification) stimulated several well designed clinical trials. GA-polymerized ragweed extract (PRW) was shown to be as effective as unmodified RW in decreasing allergy symptoms yet was both safer and more convenient. Instead of three years of conventional treatment with unmodified allergen, usually 70 injections, PRW was administered 3-15 times. Generally, 40 times less erythema and 15 times less induration occurs following such therapy. Patients so sensitive that they could not tolerate conventional immunotherapy were successfully converted to this new regime (Bacal et al., 1978; Grammer et al., 1982).

Clinical studies with GA-treated grass pollens have demonstrated

the potential applicability towards treatment of sensitivity to other inhalent allergens (Verstraeten & Wheeler, 1978). The efficiency of immunotherapy with polymerized rye grass has been shown by decreased IgE production and symptom score index evaluation (Grammer et al., 1983).

The increasing variety of GA-polymerized allergens available in Europe and North America for human immunotherapy (Patterson et al., 1981), taken in combination with greater than 70% "physician satisfaction" (Personal Communication, Bencard Allergy Service Division of the Beecham Group) suggests that the use of such preparations is likely to expand.

In spite of promising clinical results, the immunological mechanism of action of GA-polymerized antigens is unknown. The aim of this investigation was to establish the efficiency and cellular mechanism of action by which such polymers act. The study was carried out in an animal model system widely accepted as a prototype for human atopy. Specifically, it was decided to measure changes in protein antigenicity and immunogenicity occurring as a consequence of glutaraldehyde modification of ovalbumin. The consequences of treatment of inbred CBA mice with a variety of GA-modified preparations was assessed by measuring IgE, IgG and delayed hypersensitivity responses. Ultimately, the mechanism and characteristics of GA-polymerized OA induced suppression were examined at the cellular level.

MATERIALS AND METHODS

ANIMALS

Mice are CBA/J males 8-12 weeks old (Jackson Laboratories, Bar Harbor, ME.) and rats were Sprague-Dawley females, retired breeders (Charles River Canada Ltd., Ottawa, Ont.). New Zealand white rabbits, 2.5 kg, were purchased from M and P Commercial Rabbitry (Ayr, Ont.) Hartley guinea pigs (High Oaks Breeders, Toronto, Ont.) were used as a source of complement.

ANTIGENS

Crystalline bovine serum albumin (BSA) and 5x recrystallized ovalbumin (OA) were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Keyhole limpet hemocyanin (KLH) was purchased from Biomarine Pacific Supply Co., Venice, CA. Purified Ascaris suum allergen (Asc-1) was prepared according to Hussain et al (1973).

DNP-protein conjugates were prepared as previously described (Colby & Strejan, 1980). The following derivatives were prepared: DNP_3-Asc, DNP_3-OA, DNP_{18}-BSA and TNP_{75}-KLH. The subscripts refer to the average number of mols of DNP/mol of carrier, assuming a molecular weight of 17,000 for Asc-1, 40,000 for OA, 72,000 for BSA and 800,000 for the dissociated form of KLH. Protein concentrations were determined by the Lowry modification of the Folin-Ciocalteu method. (Lowry et al. 1951)

PREPARATION AND CHARACTERIZATION OF GLUTARALDEHYDE-MODIFIED-OVALBUMIN.

(i) *Formation of modified proteins:* A variety of glutaraldehyde.

(GA)-modified-OA-preparations, ranging from lightly substituted, unpolymerized ovalbumin to highly crosslinked, high molecular weight polymers, were obtained depending upon the reaction conditions selected.

OA_1-L was prepared by rapidly adding 28 λ of a 50% GA solution to 10 mls of 1 mg/ml ovalbumin (phosphate buffered saline, PBS, pH 7.0). This reaction, carried out at a 600:1 GA to OA molar ratio, was allowed to proceed at room temperature with gentle stirring for 10 minutes. OA_1-H was prepared under similar conditions except that a 10,000:1 GA to OA molar ratio was obtained by the addition of 470 λ of 50% GA to the OA solution.

OA_4/OA_{175} was prepared by adding 400 λ of a 6% GA solution dropwise over a period of three minutes to 2 ml of 25 mg/ml OA (PBS pH 7.0). The reaction mixture was gently stirred for 20 minutes and allowed to stand at room temperature for an additional 40 minutes.

For the formation of OA-POL, 50 mg OA were dissolved in 2 ml 0.2M sodium acetate-acetic acid buffer pH 5 and 0.4 ml of a 6% glutaraldehyde solution was added dropwise, at room temperature, to the OA solution over a period of 3 minutes with gentle stirring. Stirring was continued for 20 minutes and the mixture was allowed to stand at room temperature for an additional 40 minutes. The sample was then diluted by the addition of 2-3 ml borate buffered saline (BBS, pH 8.4).

All preparations were dialysed extensively against BBS to remove excess unreacted GA.

Isolation and molecular weight determination: Molecular weights were estimated by gel filtration on columns of Sephadex G-200, Sepharose 4B, Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) or Biogel A-50 m (Bio-Rad Laboratories, Richmond, CA). OA (40,000 d.), BSA (72,000 d.),

rat IgG (160,000 d.), the associated form of KLH (8 x 10^6 d.), Brome mosaic virus (4.6 x 10^6 d.) and Papaya mosaic virus (31.4 x 10^6 d.) were used as molecular weight markers on the appropriate matrices. Ratios of elution volume/void volume (Kav) were calculated and plotted against molecular weight as described at Results. For preparative purposes, the modified ovalbumin preparations were collected at the appropriate elution volumes, protein concentrations determined and were stored at -70°C until needed.

IMMUNIZATION AND TREATMENT

To induce antibody formation, groups of at least 4 mice were injected i.p. with 0.5 ml volumes containing 2 ug antigen adsorbed onto 2 mg alum, $Al(OH)_3$. Suppressive treatment with native OA or GA-modified-OA preparations was via i.p. injection in isotonic saline without adjuvant. Treatment protocols and immunization schedules are given in detail at Results.

DELAYED HYPERSENSITIVITY

Delayed hypersensitivity (DTH) was induced by injecting 100 ug OA or OA-POL in 0.2 ml CFA at the base of the tail. Thirteen days later DTH was elicited by intradermal injection into the footpad of 40 ug OA, 40 ug OA-POL or saline in 20 λ volumes. A Hamilton syringe was used. Three replicate measurements of footpad thickness were taken with an engineer's micrometer (Mitutoyo, Japan) immediately prior to, and 24 hours after, elicitation. Intensity of DTH was based upon changes in thickness of the same foot before and after elicitation (T_{24}/To), rather than comparing thickness of the challenged foot with that of an unchallenged or saline

injected contralateral foot as conventionally reported. This approach was chosen when considerable differences in thickness were found between unchallenged opposite footpads in normal mice.

ADOPTIVE TRANSFER

Donor mice were primed with 2 ug OA or TNP-KLH in alum 30 days prior to transfer or were treated with a GA-modified-OA at the times indicated at Results. On the day of transfer, animals were sacrificed, their spleens were removed aseptically, single cell suspensions were prepared, washed in Hanks balanced salts solution (BSS), counted in .04% trypan blue to determine viability and were injected in 0.5 ml volumes i.v. to either normal or irradiated syngeneic recipients. Irradiation was carried out 3-4 hours before transfer with 660 rd from a Gammacell 20 (Atomic Energy of Canada Ltd., Ottawa, Ont.). All recipients were boosted with 2 ug OA or DNP-OA in alum, 4-6 hours after cell transfer.

DEPLETION OF LYMPHOCYTE SUBOPULATIONS

Monoclonal anti-Thy 1.2, (Becton-Dickinson, Palo Alto, CA) anti-Lyt 1.1 and anti-Lyt 2.1 (Cedarlane, Hornby, Ont.) sera were used to deplete specific lymphocyte populations prior to adoptive transfer. Spleen cells at a concentration of 20 x 10^6/ml in BSS were incubated with the appropriate amount of antibody for 45 minutes at 4°C. The cells were washed once in BSS and then resuspended in BSS containing detoxified guinea pig complement at a final dilution of 1:9. Cells were incubated in a 37° waterbath for 30 minutes, washed, resuspended in BSS and recounted. Spleen cells treated with C alone were included in each experiment.

PROTEIN IODINATION

DNP-BSA and OA were labelled with $Na^{125}I$ (Amersham, Oakville, Ont.) by the chloramine T method described in Yagi et al. (1963). After labelling 500 ug protein with 0.5 mCi ^{125}I, unbound iodide was removed by Sephadex G-25 gel filtration and extensive dialysis against BBS.

ANTIBODY DETERMINATIONS

Mice were bled in equal volumes by cardiac puncture, the blood from all individuals in a group was pooled after each bleeding and the sera were stored at -20°C until needed.

Murine IgE titers were determined by passive cutaneous anaphylaxis, PCA. (Ovary, 1958). 0.1 ml serial dilutions of mouse sera in physiological saline were injected intradermally into shaved backs of S-D rats. Rats were challenged 48 hours later by i.v. injection of 2 mg DNP-BSA or OA in one ml 1% Evans blue to determine anti-hapten or anti-carrier antibodies respectively. Titers were recorded as reciprocals of the highest dilution of serum giving a blueing reaction of 5 mm diameter or greater, 15-20 minutes after challenge.

Murine IgG antibody to hapten or carrier was measured by a double antibody radioimmunoassay (Strejan et al. 1977). Anti-DNP and anti-OA antiserum standards were prepared by the ip injection of separate groups of CBA mice with 100 ug TNP-KLH or 100 ug OA in CFA at two week intervals. Quantitative precipitin tests showed the final serum pools to contain 2.54 mg/ml anti-DNP and 3.0 mg/ml anti-OA antibody. Rabbit anti-mouse IgG was prepared by monthly injection of 1 mg murine IgG in CFA. Mouse IgG was purified from serum pools by ammonium sulfate precipitation and DEAE ion exchange chromatography. Its purity was determined by

immunoelectrophoresis.

COMPETITIVE INHIBITION RADIOIMMUNOASSAY

A standard hyperimmune mouse anti-OA serum was diluted serially in 0.5 ml volumes in the presence of 0.5 ug ^{125}I-labeled OA and the mixture was incubated for 30 minutes at 37°C and 20-40 hours at 4°C. After precipitation of the immune complexes with excess rabbit anti-mouse IgG, the precipitates were washed and the radioactivity in the precipitates was used to calculate the extent of binding. The amount of antibody required to bind 74% of the labeled antigen was then used for the inhibition studies. A constant amount of antibody in 0.5 ml volumes was incubated with 0.5 ug ^{125}I-OA and with increasing amounts of native OA or of OA-POL in 0.5 ml, for 30 minutes at 37° and 20-40 hours at 4°C. The immune complexes were precipitated and radioactivity was determined as described above.

STATISTICAL ANALYSIS

PCA titers were log-transformed and the geometric means from replicate experiments determined. Single factor analyses of variance with multiple comparisons of means were used to determine statistical significance. Molecular weight determinations and radioimmunoassays were calculated using linear regression analysis.

RESULTS

I Preparation and Characterization of Glutaraldehyde-Modified Ovalbumin

(i) Formation and Isolation by Gel Filtration

Depending upon the reaction conditions selected, a wide variety of GA-modified proteins can be obtained. See Table I for summary and nomenclature. OA_1-L and OA_1-H, prepared under conditions designed to minimize polymer formation, were collected at the same elution volume from Sephadex G-200 columns as the major peak for native ovalbumin (Figure 1(i)). A highly heterogeneous mixture of OA polymers, designated OA_4/OA_{175}, was prepared at pH 7.0 using 25-fold higher protein concentrations than above. Polymers ranged in molecular weight from 160,000 d. to almost 10^7 d. For some experiments this preparation was subdivided into one of average molecular weight 160,000 d, designated OA_4, and one of average molecular weight 7×10^6 d, designated OA_{175} (See figures 2,3).

Polymer formation at pH 5.0, the isoelectric point of ovalbumin, gave the preparation designated OA-POL. Its molecular weight, as estimated by gel filtration on Biogel A-50 m (Figure 1(ii), 4) was approximately 35×10^6 d. Arithmetically this corresponds to a polymer of approximate molecular formula of OA_{875}.

(ii) Antigenic crossreactivity between OA and GA-modified OA

The extent of crossreactivity between native and chemically modified ovalbumin, both monomeric and polymerized forms, was determined by a competitive inhibition RIA for anti-OA IgG and by PCA for anti-OA IgE antibodies.

IgG anti-OA: It can be seen from Figure 5 that, as expected, 0.5 ug native OA were required to inhibit by 50% the binding between a known

TABLE I

Preparation and Isolation of Glutaraldehyde-modified Ovalbumin

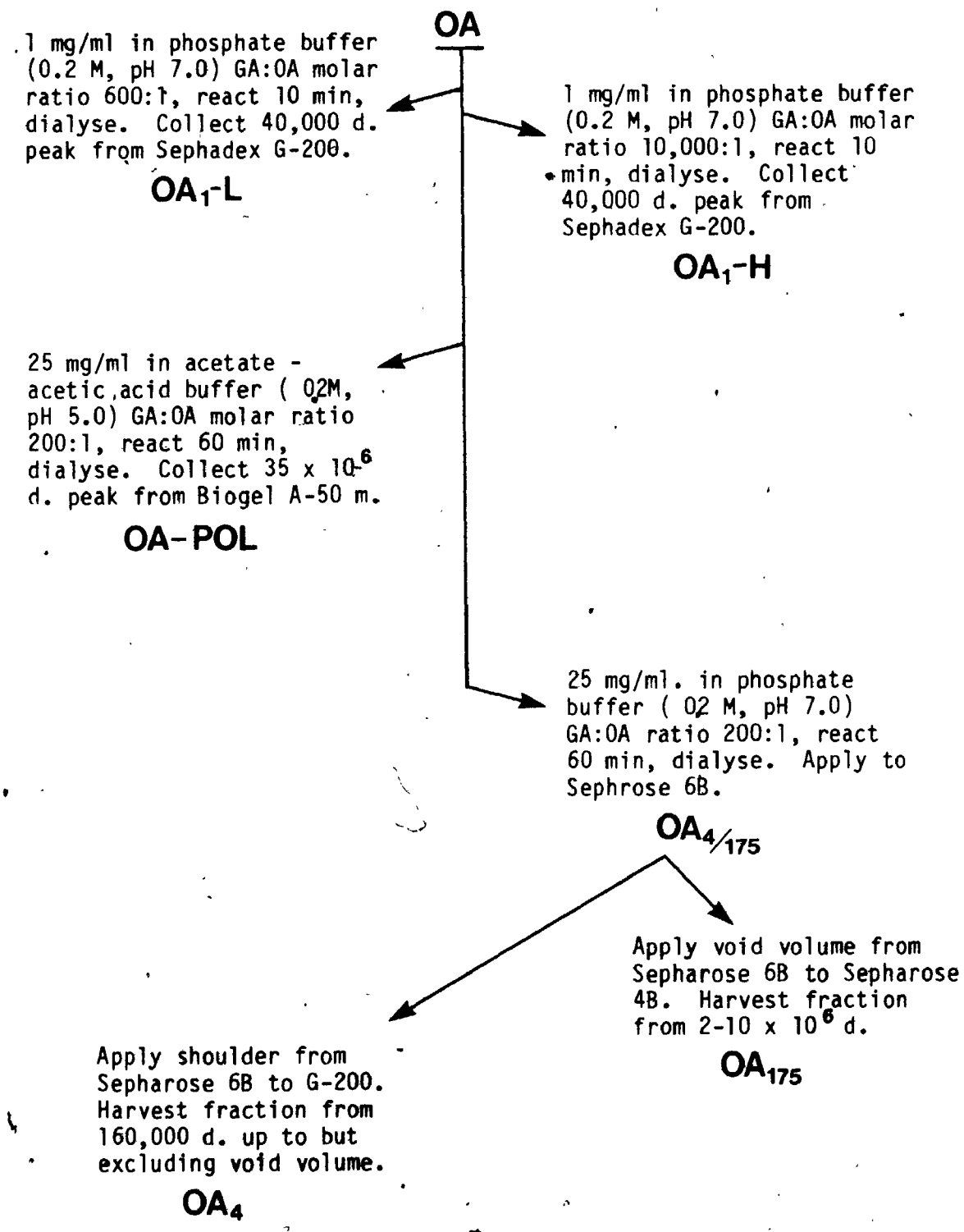


FIGURE 1(i): Elution of OA_1-L and OA_1-H. Four mls of 1 mg/ml OA_1-L (●) or OA_1-H (○) were applied to separate 2.5 x 82 cm Sephadex G-200 gel filtration columns equilibrated in borate saline pH 8.4. The fraction corresponding to the elution volume of OA (▲) (282-330 mls) was collected for further use. Vo, void volume; Ve, elution volume.

FIGURE 1(ii): Elution profile of OA-POL. One ml containing approximately 25 mg. OA-POL (●) was applied to a 1.5 x 80 cm Biogel A-50 m column equilibrated in borate saline pH 8.4. The fraction eluting from 90-105 ml was pooled and designated OA-POL.

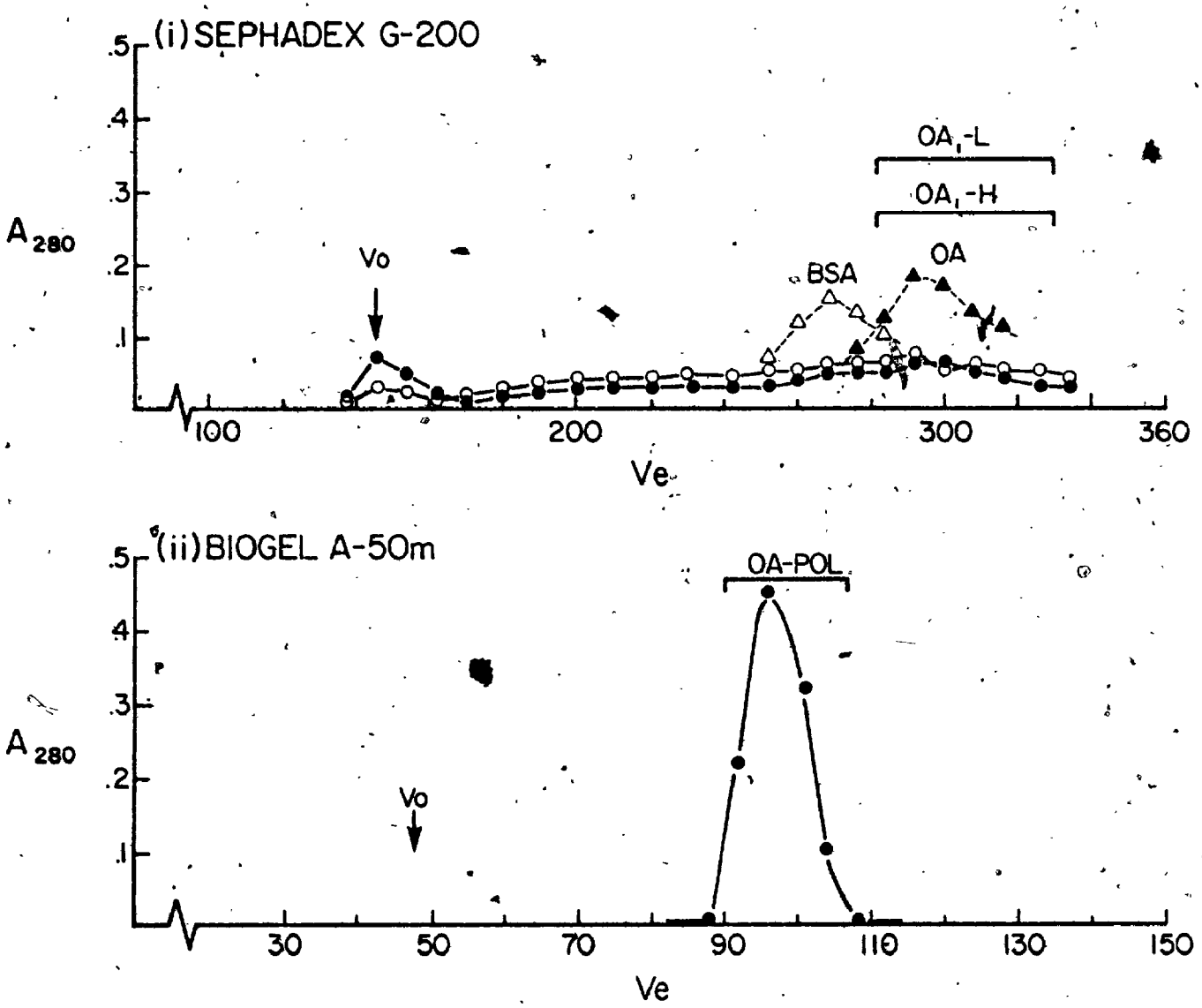
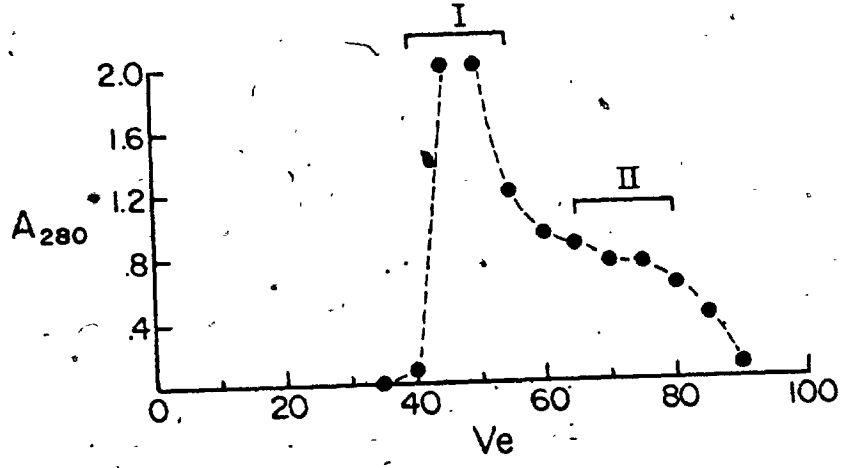

(i) SEPHADEX G-200
A280
.5
.4
.3
.2
.1
Vo
BSA
OA
OA1-L
OA1-H
100
200
300
360
Ve
(ii) BIOGEL A-50m
A280
.5
.4
.3
.2
.1
Vo
OA-POL
30
50
70
90
110
130
150
Ve

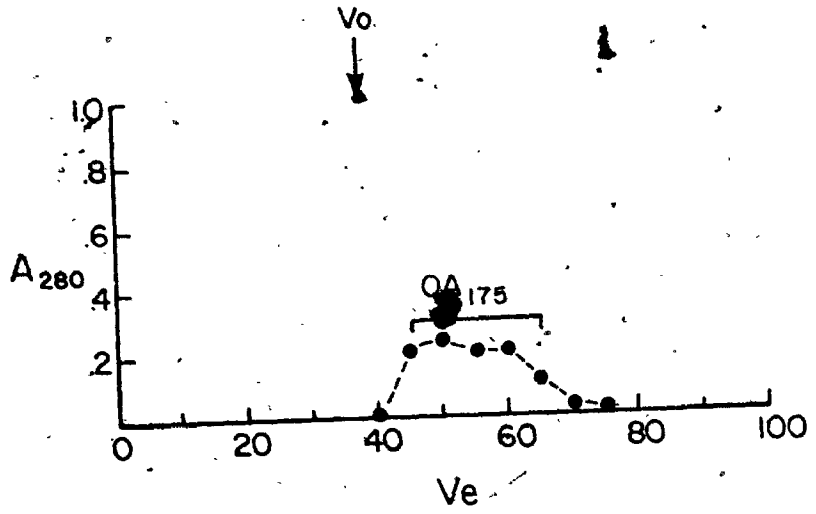
FIGURE 2: Elution profile of $OA_{4/175}$. One ml containing approximately 25 mg was protein applied to a 1.5 x 80 cm Sepharose 6B column equilibrated with borate saline (Figure 2i). The entire elution volume from 40-80 ml was collected for further use as $OA_{4/175}$. The void volume (I) was collected and applied to a 1.5 x 80 cm Sepharose 4B column (Figure 2ii) to obtain the fraction eluting from 45-65 mls, OA_{175}. The shoulder from Sepharose 6B (II) was collected and applied to a Sephadex G-200 column (Figure 2iii). The fraction eluting from 180-210 ml was designated OA_4.

Effective ranges for these media, as provided by the manufacturer, are as follows: Sephadex G-200, 10^4-2 x 10^5d; Sepharose 6B, 10^5-2 x 10^6d; Sepharose 4B, 10^5-10 x 10^6d. Vo, void volume; Ve, elution volume.

(i) SEPHAROSE 6B



(ii) FRACTION I ON SEPHAROSE 4B



(iii) FRACTION II ON SEPHADEX G-200

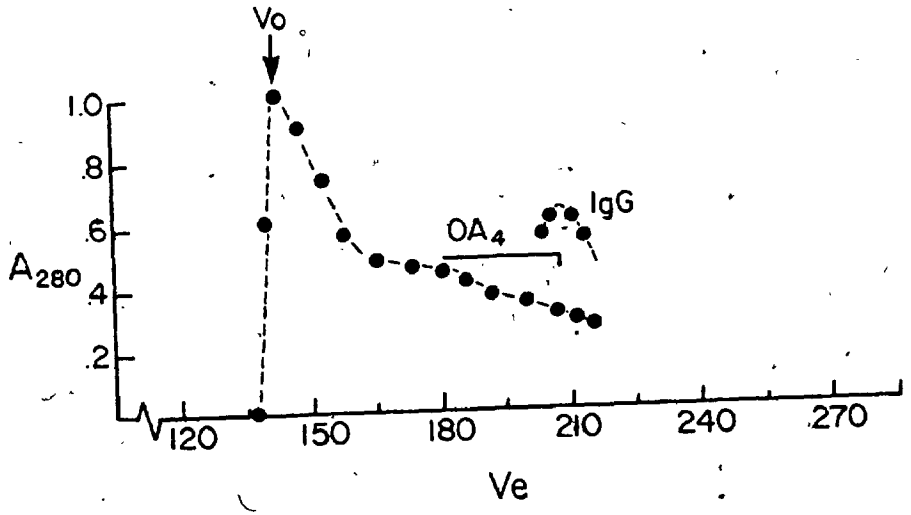


FIGURE 3: Molecular weight determination of OA_1-L, OA_1-H, and OA_4. 5 mg protein in 1.0 ml volumes were applied to 1.5 x 80 cm Sephadex G-200 columns equilibrated with borate saline (0.2 M, pH 8.4), ovalbumin (40,000) rat IgG (165,000 d.) were used as molecular weight markers. Kav: ratios of the elution volume/void volume.

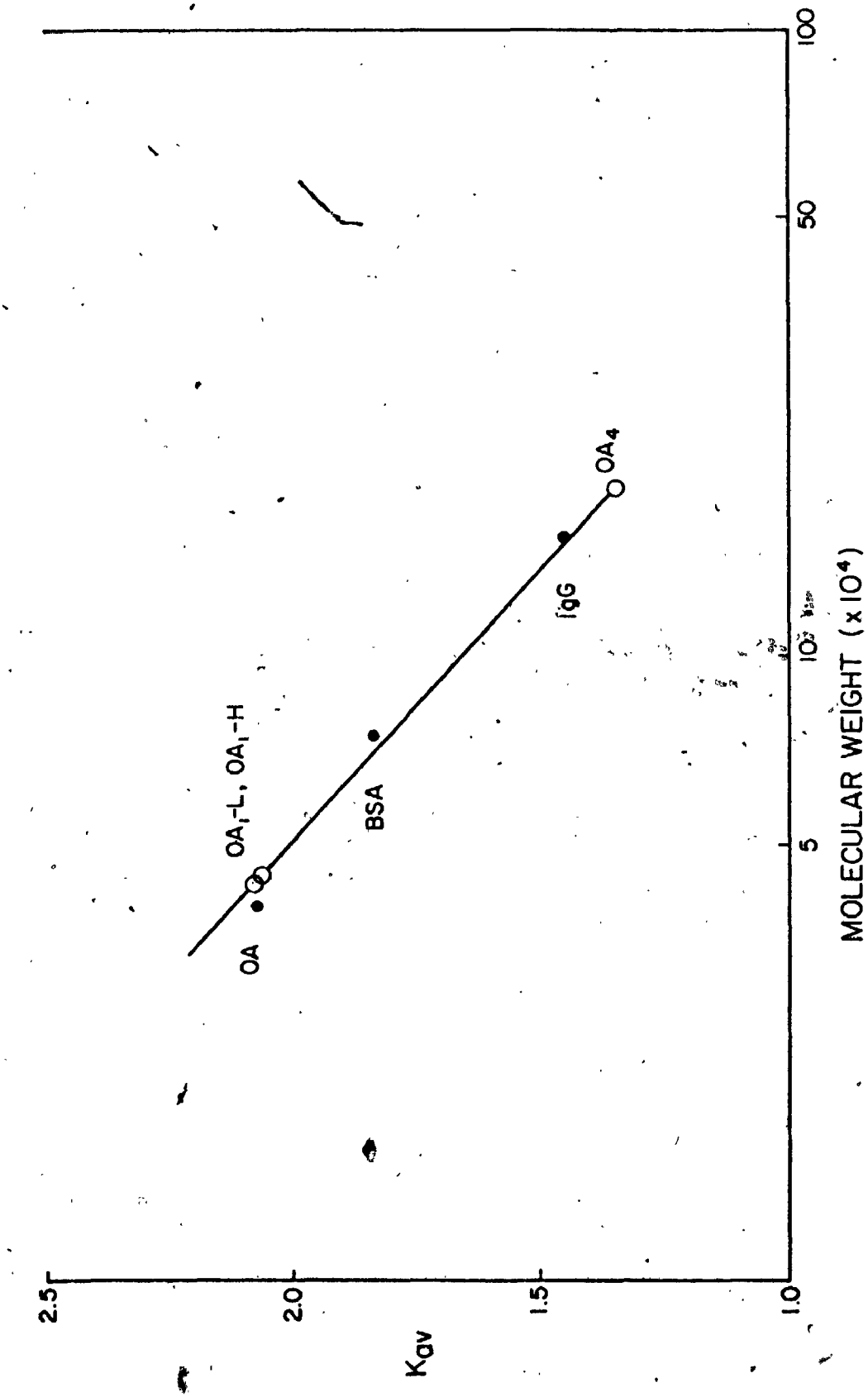
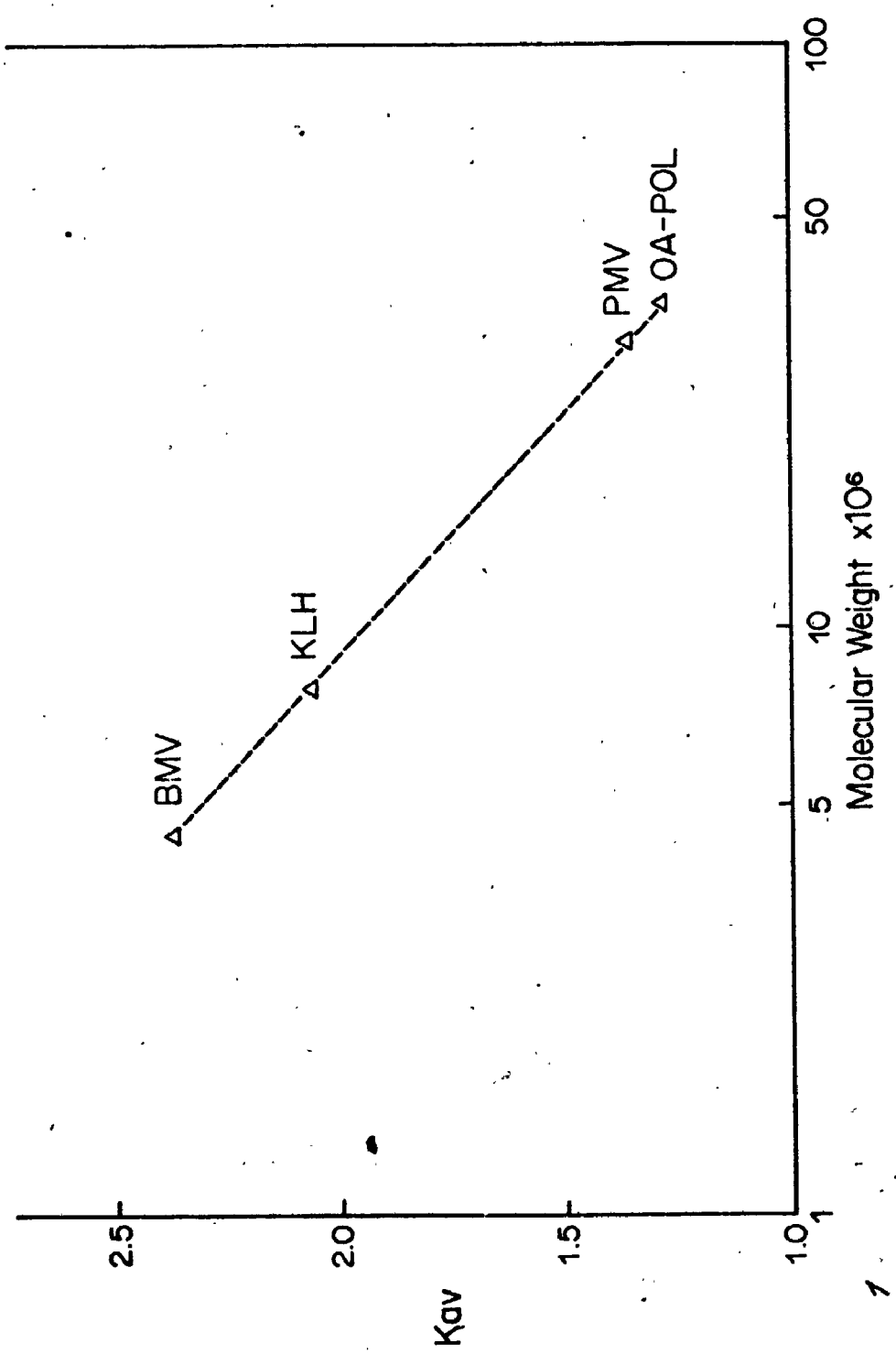

2.5
2.0
1.5
1.0
Kav
100
50
5
MOLECULAR WEIGHT (x 10⁴)
OA
OA1-L, OA1-H
BSA
IgG
OA4

FIGURE 4: Molecular weight determination of OA-POL Five mg OA-POL in 1.0 ml volumes were applied to a 1.5 x 80 cm Biogel A-50m column equilibrated with 0.05 M phosphate 0.15 M NaCl buffer (pH 6.5). Brome mosaic virus (BMV), Papaya mosaic virus (PMV) and keyhole limpet hemocyanin (KLH) were used as molecular weight markers. Kav = Ratios of the elution volume/void volume.


BMV
KLH
PMV
OA-POL
2.5
2.0
1.5
1.0
Kav
1
5
10
50
100
Molecular Weight x10⁶

amount of anti-OA IgG antibodies and 0.5 ug ^{125}I-labeled native OA. Thus OA and ^{125}I-labeled OA are 100% crossreactive with respect to anti-OA IgG antibodies. On the other hand, twice as much OA_1-L is required for the same degree of inhibition indicating a 50% loss of antigenic crossreactivity in this GA-modified preparation. OA_1-H, OA_4/OA_{175} and OA-POL all display 80-90% decreases in antigenic crossreactivity.

These results suggest that increased chemical modification rather than polymerization is the major factor determining decreased antigenicity.

IgE anti-OA: The extent of antigenic crossreactivity between native and modified OA with respect to anti-OA antibodies of the IgE class was determined by their relative capacities to react with mast cell-bound IgE and trigger positive PCA reactions.

Thirty-four sera of anti-OA titers ranging between 1:40 and 1:6000 were titrated in the skin of separate groups of rats using, for challenge the antigens shown in Figure 6. A 75% decrease in antigenic crossreactivity was found in GA-modified but unpolymerized molecules (OA_1-H) with only an additional 10-15% decrease following increases in molecular weight approaching three orders of magnitude (OA-POL). This 90% decrease in PCA reactivity correlates well with the observations made with IgG anti-OA antibodies.

Two additional observations (not shown) suggest that a loss of native OA antigenic determinants has occurred as a consequence of glutaraldehyde modification. Increases in the amount of antigen used for PCA challenge from 1 mg to 7 mg had no effect upon the titres obtained, indicating that a simple shortage of antigen is not responsible for the decreased PCA titers found. Additionally, administration of a second PCA challenge (OA)

FIGURE 5: Degree of cross-reactivity between native OA and GA-modified ovalbumin with respect to IgG antibodies as measured by competitive inhibition radioimmunoassay. A hyperimmune mouse anti-OA serum (3.0 mg/ml) diluted 1:800 was incubated with 0.5 ug ^{125}I-labeled OA and with increasing amounts of unlabeled native OA, OA_1-L(○); OA_1-H(▲), OA_4/OA_{175}(△) or OA-POL (●), for 30 min at 37°C and for 20-40hr at 4°C. The immune complexes were precipitated with rabbit anti-mouse immunoglogulin. The lines were fitted by linear regression analysis.

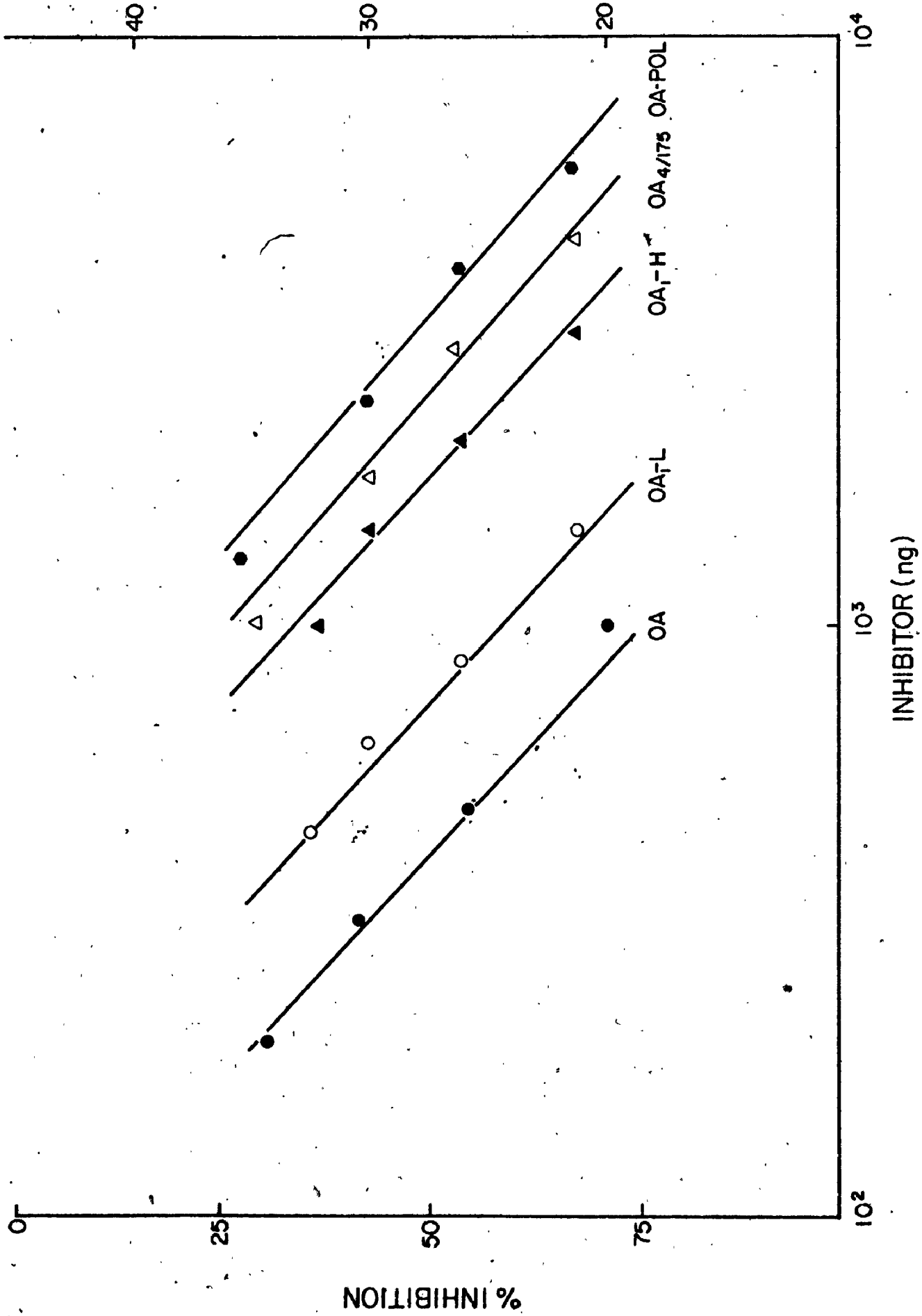
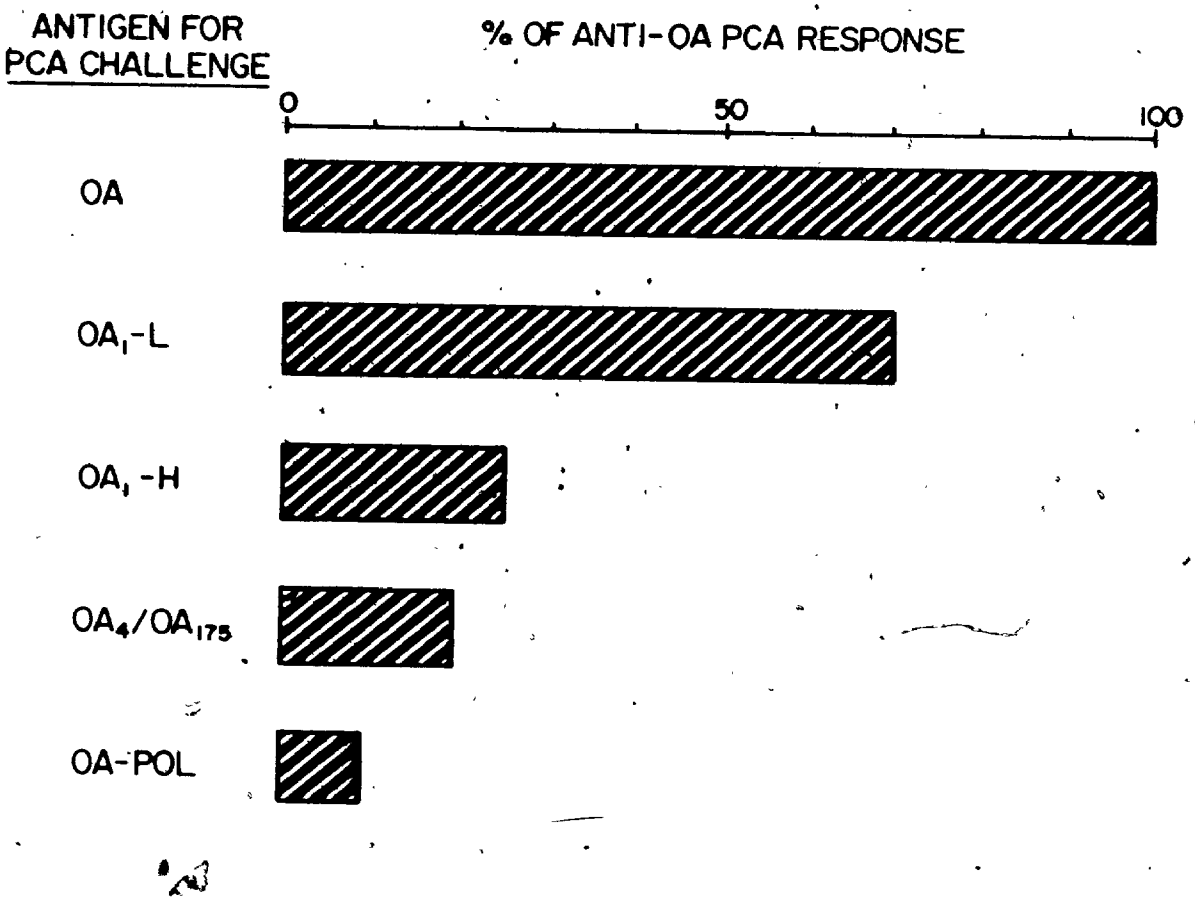

% INHIBITION
0
25
50
75
INHIBITOR (ng)
10^2
10^3
10^4
^{125}I-OA (cpm x 10^{-3})
40
30
20
OA
OA$_1$-L
OA$_1$-H^{-1}
OA$_{4/175}$
OA-POL

FIGURE 6: Crossreactivity between native and glutaraldehyde - modified ovalbumin with respect to IgE antibodies. Thirty-four sera of known anti-OA PCA titer (from 1:40 to 1:6000) were titrated on separate groups of rats. The geometric mean of anti-OA PCA titers is 100%. The decreases in PCA titer obtained after challenge with various modified OA preparations are shown as percent of control.

ANTIGENIC CROSSREACTIVITY: IgE ANTI-OA ANTIBODIES



to a rat challenged with OA-POL forty minutes previously, resulted in 100% of the expected anti-OA titer, indicating that OA-POL is not sterically hindering the reaction between mast cell-bound anti-OA IgE and native OA antigenic determinants.

Taken collectively, the experiments on crossreactivity indicate that OA-POL has strikingly reduced ability to bind antibody and that for both IgE and IgG antibody classes this represents approximately 10% of the reactivity displayed by the same antibodies towards the native molecule.

DTH anti-OA : The decreased capacity of GA-modified OA to react with anti-OA IgE and IgG antibodies was not paralleled by a similarly decreased capacity to react with OA-specific T cells. Table II shows that OA-POL is at least as good as native OA in eliciting anti-OA DTH responses of OA (CFA) primed mice. The loss of B cell (Ig)-reactive determinants does not appear to be reflected as decreased T cell reactivity.

Relative immunogenicities of OA and GA-modified OA

The question of whether OA-POL's decrease in ovalbumin antigenic determinants is also reflected as decreased immunogenicity was addressed as follows. Groups of mice were immunized with 2 ug OA or OA-POL in alum twice at 28 days interval and the 14 and 35 day sera were tested in PCA against both native OA and OA-POL. The results of Table III show that even when injected with adjuvant under conditions optimal for the induction of an IgE response, OA-POL displayed a significantly reduced ability to stimulate primary IgE antibody responses (mean PCA titers of 389 and 27 for native OA and OA-POL respectively). The secondary response was less affected. In addition, the results in Table III confirm that IgE antibodies produced after both primary and secondary stimulation reacted in PCA 20-40 fold less after challenge with OA-POL than after challenge

TABLE II

Relative capacity of OA and OA-POL to elicit delayed hypersensitivity

IMMUNIZATION[1]	DTH CHALLENGE[2]	FOOTPAD SWELLING[3] (±S.E.M.)
OA (CFA)	OA	1.15 ±.04
OA (CFA)	OA-POL	1.33 ±.06
OA (CFA)	Saline	1.01 ±.03

[1] Groups of 5 mice were immunized by s.c. injection at the base of the tail with 100 ug OA emulsified in 0.2 ml CFA on day 0.

[2] Pyrogen free saline, 40 ug OA or 40 ug OA-POL was injected as 20 ul aliquots sc into murine footpads on day 13.

[3] Ratio of footpad thickness at 24 h. vs. immediately prior to challenge (T_{24}/T_0).

TABLE III

Immunogenicity of native OA and OA-POL with respect to IgE antibody

Immunization[1]	PCA titer		
	Day	antigen challenge[2]	titer[3]
OA	14	OA	389
		OA-POL	20
	35	OA	2,308
		OA-POL	53
OA-POL	14	OA	27
		OA-POL	8
	35	OA	431
		OA-POL	80

[1] Mice were immunized with 2 ug OA or OA-POL in alum on days 0 and 28.
[2] PCA challenge was with 2 mg OA and with 2 mg or 7 mg OA-POL.
[3] Geometric mean of PCA titers of at least 3 separate groups of mice.

with native OA (mean PCA titers of 20 vs 389 and 53 vs 2308).

II. Suppression of IgE Antibody Responses

Pretreatment with GA-modified-OA

In order to determine whether OA-POL can interfere with the induction of IgE antibody responses, groups of mice were pretreated with various amounts of OA-POL in 1, 2 or 3 doses according to the schedule presented in Table IV. Control animals received 0.5 ml isotonic saline i.p. On days 0 and 28 all animals received 2 ug DNP-OA in alum i.p.. PCA titers of sera obtained 14 days after priming and 7 days (day 35) after booster are shown.

It can be seen that a single injection of 25 ug OA-POL, given 14 days before standard immunization with DNP-OA, suppressed both primary and secondary IgE responses. The extent of suppression increased significantly with the number of injections and amount of OA-POL administered. A further increase in dosage up to 1500 ug OA-POL had no additional suppressive effect (not shown).

The question next arose as to whether treatment with equivalent amounts of native OA would have the same effect. In addition we wished to ascertain the relevant molecular characteristics of GA-modified OA in terms of its ability to suppress anti-OA IgE responses. Thus, groups of mice were pretreated with 80 ug OA, 80 ug of a modified OA or with saline on days -14,-12,-10 and challenged with 2 ug DNP-OA in alum on days 0 and 28.

Table V shows that while mice pretreated with OA_{175} or OA-POL had strongly suppressed anti-DNP and anti-OA IgE levels, the animals receiving an equivalent amount of native OA, OA_1-L, OA_1-H or OA_4 were suppressed

TABLE IV

Suppression of the IgE antibody response by various amounts of OA-POL administered before immunization

Treatment[1]	Day[2]	PCA titer[3]			
		day 14		day 35	
		a-DNP[4]	a-OA[5]	a-DNP	a-OA
Saline	-14,-12,-10	800	160	8,000	4,000
OA-POL 25 ug	-14	320	40	800	800
OA-POL 25 ug	-14,-12	80	20	800	200
OA-POL 25 ug	-14,-12,-10	40	10	800	400
OA-POL 80 ug	-14,-12,-10	10	10	400	160

1 Groups of 4 mice were injected i.p. with saline or with OA-POL in saline and were immunized on days 0 and 28 with 2 ug DNP-OA in alum.
2 Day of pretreatment with OA-POL.
3 Expressed as the reciprocal of the highest serum dilution giving a skin reaction of least 5 mm diameter.
4 Anti-DNP titers following challenge with 2 mg DNP-BSA in 1 ml 1% Evans' blue solution.
5 Anti-OA titers following challenge with 2 mg OA in Evans' blue solution.

TABLE V

Effects of treatment with glutaraldehyde-modified ovalbumin administered before DNP-OA immunization

TREATMENT	anti-DNP			anti-OA		
	day 7 IgE[2]	14 IgE [IgG][3]	35 IgE [IgG]	day 7 IgE	14 IgE [IgG]	35 IgE [IgG]
Saline	<10	800 [16]	2,000 [262]	<10	160 [12]	800 [165]
OA	60	80*[5]	1,500 [92]	200	200 [79]	800 [286]
OA_1-L	50	80*[6]	900 [71]	100	120 [32]	400 [197]
OA_1-H	50	80*[6]	900 [156]	200	120 [40]	400 [205]
OA_4	100	180*[7]	1,300 [144]	160	160 [164]	500 [247]
OA_{175}	100	150*[<5]	500*[131]	50	40* [181]	160*[261]
OA-POL	60	30*[5]	320*[199]	80	20* [180]	80*[254]

* Significantly suppressed relative to saline-treated control ($p<0.05$ or smaller)

[1] Three or four groups of 4 mice each were treated with saline or 80 ug protein on days -14,-12,-10 and were immunized on days 0 and 28 with 2 ug DNP-OA in alum.

[2] PCA titer is expressed as the reciprocal of the highest serum dilution giving a skin reaction of at least 5 mm diameter following challenge with 2 mg DNP-BSA or 2 mg OA 1% Evans blue solution.

[3] Micrograms specific antibody/ml serum.

only with respect to anti-hapten antibodies and not with respect to anti-OA. When the results were subjected to a single factor analysis of variance and the statistical significance between treatment groups was compared, it was seen that even in the case of anti-DNP titers, the group receiving OA-POL were better suppressed than the groups treated with native OA.

Of interest was the observation that a short burst of IgE production, indicative of Th cell activity was found prior to Ts induction in all but the saline injected group. IgG anti-OA responses were significantly higher in all treated groups regardless of whether the IgE response was suppressed or not. IgG anti-hapten responses did not appear to be significantly different between saline controls and groups displaying suppression of IgE responsiveness.

Antigenic-specificity of OA-POL-induced suppression

The antigenic specificity of OA-POL-induced suppression was determined by treating groups of mice with saline or 80 ug OA-POL on days -14,-12,-10 followed by immunization with 2 ug TNP-KLH in alum. The resulting PCA titers in both groups were identical (Table VI). These results demonstrated that suppression was carrier-specific. In a separate experiment, cotransfer of 10^7OA (alum) and 2 x 10^7 TNP-KLH (alum)-primed cells with 6 x 10^7 spleen cells from normal or OA-POL treated mice, followed by boosting of the recipients with 2 ug TNP-KLH (in alum) resulted in anti-TNP responses that were the same in groups getting OA-POL cells or normal spleen cells. This further demonstrates the antigenic specificity of OA-POL-induced suppression.

TABLE VI

Antigenic specificity of OA-POL-induced suppression

TREATMENT[1]	IMMUNIZATION[2]	Anti-DNP PCA titer at day 14	Anti-DNP PCA titer at day 21
Saline	DNP-OA	400	320
OA-POL	DNP-OA	40	40
Saline	TNP-KLH	20	20
OA-POL	TNP-KLH	20	20

[1] Mice were treated with saline or 80 ug OA-POL on days -14, -12, -10.

[2] Mice were immunized with 2 ug DNP-OA or 10 ug TNP-KLH in alum on day 0 and bled 14 and 21 days later.

Effects of pretreatment upon delayed hypersensitivity responses

The effect of pretreatment with GA-modified OA upon cell mediated immunity as measured by delayed hypersensitivity was examined. Mice were pretreated in the standard fashion with saline or 80 ug protein injections on days -14,-12,-10. On day 0 all groups were primed with 100 ug OA in CFA. The effects of pretreatment upon DTH were assessed by footpad swelling 13 days later with an i.d. injection of 40 ug OA or OA-POL. Footpad swelling is expressed as the ratio of the thickness at 24 hours with that at time zero in the same foot (T_{24}/T_0). The conventional method comparing swelling in the test foot to that of the contralateral foot was not used as it was felt to be insufficiently accurate.

It can be seen that at day 14, the time of peak primary IgE responsiveness, pretreatment with native OA, modified but unpolymerized OA, or very high molecular weight OA polymers did not result in either significant enhancement or suppression of delayed hypersensitivity (Table VII). Intradermal injection of saline for challenge resulted in minimal nonspecific swelling (T_{24}/T_0=1.01).

The results of this section indicate that an optimal series of pretreatment injections result in up to 90% suppression of IgE responsiveness. Suppression is antigen-specific and occurs without concomitant suppression of IgG antibody responses or delayed hypersensitivity. Suppressive efficiency correlates well with increasing molecular weight of the treatment preparation used.

Isotype-specific abrogation of ongoing IgE responses

To examine whether suppression occurs under conditions in which treatment is administered after the induction of IgE responses, groups of

TABLE VII

Effect of pretreatment with OA, OA_1-H or OA-POL upon delayed hypersensitivity responses.

IMMUNIZATION[1]	IMMUNIZATION[2]	FOOTPAD SWELLING[3] (±S.E.M.)
Saline	OA (CFA)	1.28 ± .03
OA	OA (CFA)	1.26 ± .02
OA_1-H	OA (CFA)	1.29 ± .03
OA-POL	OA (CFA)	1.29 ± .03

[1] Groups of 5 to 16 mice were treated with saline or 80 ug protein on days -14,-12,-10.

[2] Immunization consisted of a sc injection at the base of the tail on day 0 with 100 ug OA emulsified in 0.2 ml CFA.

[3] Mice were challenged on day 13 with pyrogen free saline or 40 ug OA-POL in 20 ul. The ratio of footpad swelling at 24 h vs. immediately prior to challenge is given (T_{24}/T_0). Challenge with 40 ug OA showed all 4 groups to be equally similar.

mice received the standard dose of 2 ug OA in alum and were then treated at various times thereafter with one course of 3 OA-POL injections. All animals were boosted with 2 ug OA in alum 10 or 20 days after the end of treatment. The results are presented in Table VIII. It can be seen that neither primary nor secondary anti-OA PCA titers were affected by the treatment. On the other hand, IgG anti-OA antibodies were significantly increased in all treated groups when compared to the saline-injected controls.

In a subsequent experiment, animals were treated with two courses of 3 injections of OA-POL or native OA. Each injection consisted of 80 ug antigen in isotonic saline. Figure 7 shows that treatment with OA-POL resulted in an 8-10-fold decrease in PCA titers compared to the saline control. It is important to note that not only was the suppression maintained throughout six additional boosters with 2 ug OA (alum) over a period of at least 321 days but also that the anamnestic response observed in the saline-treated group was absent in OA-POL treated groups. Initially, treatment with native OA was equally effective. However by day 161, following the third OA (alum) booster, suppression in OA-treated animals had faded to non-significant levels.

Anti-OA IgG antibodies were increased in both treatment groups (moreso for OA-treated than for OA-POL-treated groups) when compared to the saline control. The same pattern of suppressed IgE and increased IgG antibody levels was found in 3 separate experiments.

III. Cellular Interactions in OA-POL-Induced Suppression

Adoptive transfer of suppression

Donor mice were treated i.p. with OA-POL, OA_1-H, OA or were left

TABLE VIII

Failure to abrogate the IgE anti-OA response by one course of treatment

Treatment[1]	Day	Primary Response		Secondary Response	
		IgE[2]	IgG[3]	IgE	IgG
Saline	5,7,9	400	21	1200	34
OA-POL	5,7,9	200	90	800	810
OA-POL	14,16,18	400[4]	190	600	1150
Saline	28,30,32	ND[5]	ND	1000[6]	225
OA-POL	28,30,32	ND	ND	1000[6]	3450

1 Mice were primed with 2 ug OA in alum i.p. on day 0 and were boosted with the same amount 28 days later. Treatment with saline or 80 ug OA-POL was given i.p. on the days indicated. Animals were bled on day 14 (primary response) and day 35 (secondary response) except where indicated.

2 PCA titer.

3 Expressed in ug/ml anti-OA antibody.

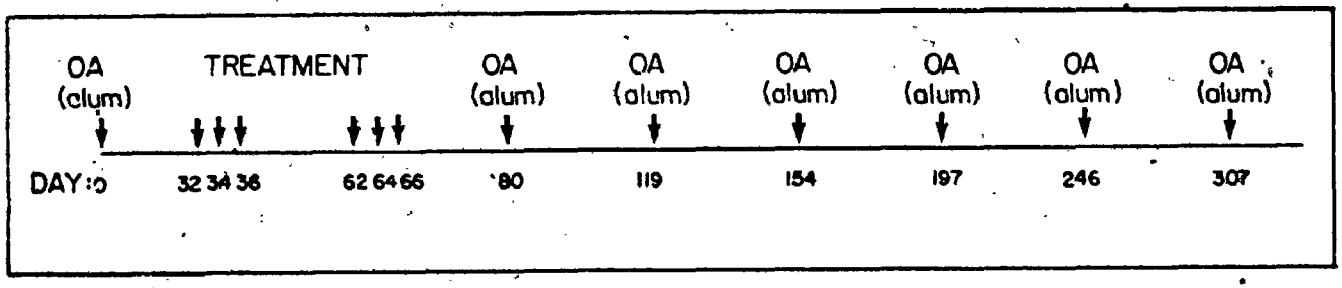
4 Day 21 sera.

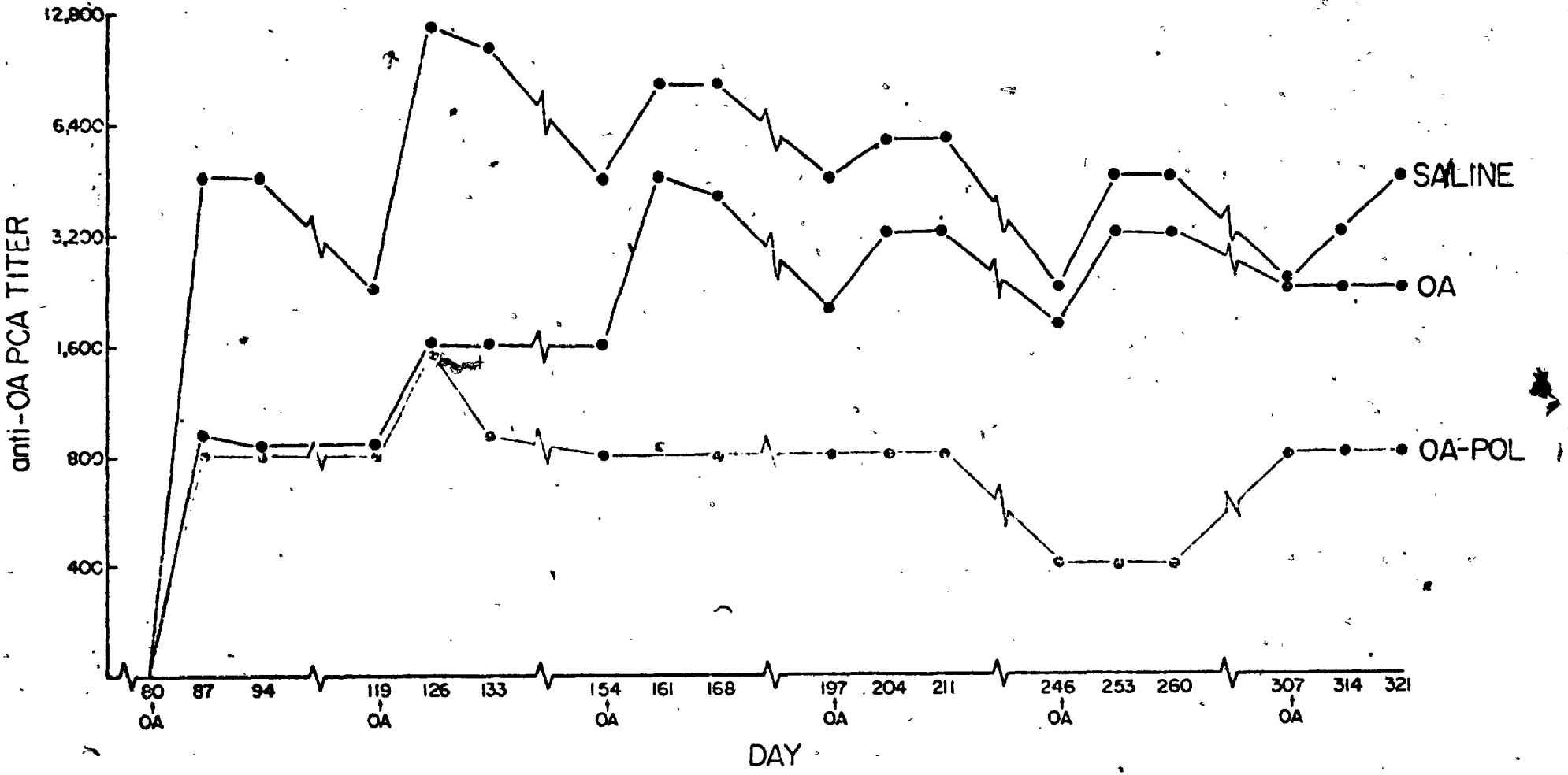
5 Not determined.

6 Mice were boosted with OA (alum) on day 32 instead of day 28.

FIGURE 7: Abrogation of an established IgE response after two courses of treatment. Groups of mice were immunized with 2 ug OA in alum on days 0, 80, 119, 154, 197, 246 and 307. Treatment with 80 ug OA-POL, 80 ug native ovalbumin, or saline in two courses of 3 injections each was administered as shown in the inset.

LONGLIVED ABROGATION OF ESTABLISHED IgE RESPONSES





untreated, and 70 x 10^6 donor spleen cells were transferred to normal, nonirradiated recipients 10 days after the termination of treatment. The recipients were primed on the same day and were boosted 28 days later with 2 ug DNP-OA in alum. IgE and IgG antibody levels were determined on day 7, 14 and 35. The results presented in Table IX show that mice receiving cells from OA-POL-treated donors had greatly decreased PCA-titers during both primary and secondary responses while recipients injected with normal spleen cells or those from OA_1-H or native OA treated donors had IgE levels similar to those encountered in normal animals under the same immunization conditions. These results clearly indicate that spleen cells from mice treated with OA-POL have the capacity to interfere with the production of IgE anti-hapten and anti-carrier antibodies in normal animals.

Thymus-lymphocyte dependence of OA-POL-induced suppression

The previous findings were confirmed and further expanded with adoptive transfer experiments in which spleen cells from OA-POL-treated donors were cotransferred with cells from donors primed with 2 ug OA in alum to x-irradiated recipients. Before transfer, an aliquot of cells from OA-POL treated animals was treated with anti-Thy 1.2 serum and C. After transfer, the recipients were boosted with 2 ug OA in alum and the day 14 anti-OA IgE and IgG antibodies were determined. Figure 8 shows that recipients of OA-primed cells gave the expected adoptive secondary IgE response (PCA titer 2000), while cotransfer of 60 x 10^6 OA-POL cells with 20 x 10^6 OA cells significantly suppressed the ability of the OA-primed cells to mount a secondary IgE response in the recipient (PCA titer 400). In preliminary experiments (not shown) it was established that

TABLE IX

Adoptive transfer of spleen cells from OA-POL-treated donors to normal recipients

Donor Treatment[1]	Recipient[2] PCA anti-DNP day 7	14	35	anti-OA day 7	14	35
Saline	<10	400	2000	<10	100	400
OA	200	400	1600	50	80	400
OA_1-H	100	200	1600	50	80	800
OA-POL	80	80	400	20	10	40

[1] Donor mice were pretreated by ip injection of saline, or 80 ug OA, OA_1-H or OA-POL 14, 12 and 10 days prior to adoptive transfer.

[2] Syngeneic, normal, non-irradiated recipients were injected iv with 70 x 10^6 donor spleen cells. They were boosted the same day (day 0) and 28 days later with 2 ug DNP-OA in alum ip.

normal spleen cells did not interfere with the ability of OA-primed cells to mount a secondary IgE response. Treatment of cells from OA-POL donors with anti-Thy 1.2 serum and C abrogated the suppression while treatment with C alone was without effect. In contrast, anti-OA IgG antibody levels remained constant regardless of the combination of cells transferred. These results demonstrate that the suppression is mediated by T lymphocytes and strongly suggest that its effect is restricted to the IgE class. Moreover they indicate that the suppressor cells interferred not only with the stimulation of a primary IgE response as shown in Table IX but also with the ability to display a secondary IgE response, by spleen cells that have been already primed (Figure 8). Analogous experiments (Figure 9) demonstrate that the abrogation of well established, ongoing anti-OA IgE responses is also T cell mediated.

Lyt 1.1 cell dependence of suppression

The Lyt phenotype of OA-POL induced T suppressor cells was investigated as shown in Figure 10. OA-POL treated and OA (alum)-primed cell donors were prepared as previously described 5 x 10^7 OA-POL cells were treated with monoclonal anti-Lyt 1.1 or monoclonal anti-Lyt 2.1 antibodies plus complement and transferred along with 10^7 OA-primed cells into irradiated recipients that were then challenged with 2 ug ovalbumin (alum). The geometric mean of day 14 antibody concentrations obtained from duplicate experiments are shown. The number of cells remaining in the Lyt 1-depleted and the Lyt 2-depleted populations was well within conventionally accepted values (56%, 82% viability). Recipients of the OA-POL Lyt 2-depleted population demonstrated the same degree of suppression as recipients of untreated OA-POL donor cells. OA-POL cells depleted of Lyt 1 populations lost their ability to suppress IgE responses. IgG responses were neither suppressed nor enhanced.

FIGURE 8: Demonstration by coadoptive transfer of thymus dependence and IgE class-specificity of OA-POL-induced suppression. 60 x 10^6 spleen cells/recipient, from OA-POL-injected donors were treated with monoclonal anti-Thy 1.2 serum and C, Complement alone, or were left untreated. These normal spleen cells were mixed with 20 x 10^6 spleen cells from OA-primed donors and were injected i.v. to X-irradiated (660 rad) recipients. The recipients were boosted with 2 ug OA in alum on the day of transfer. Day 14 anti -OA IgE as PCA titers (▨) and anti-OA IgG as ug/ml (□) are shown.

T CELL DEPENDENCE AND IgE CLASS SPECIFICITY OF SUPPRESSION

DONOR CELLS	
60×10^6	20×10^6
NORMAL	OA
OA-POL	OA
OA-POL (a-θ +C)	OA
OA-POL (C)	OA

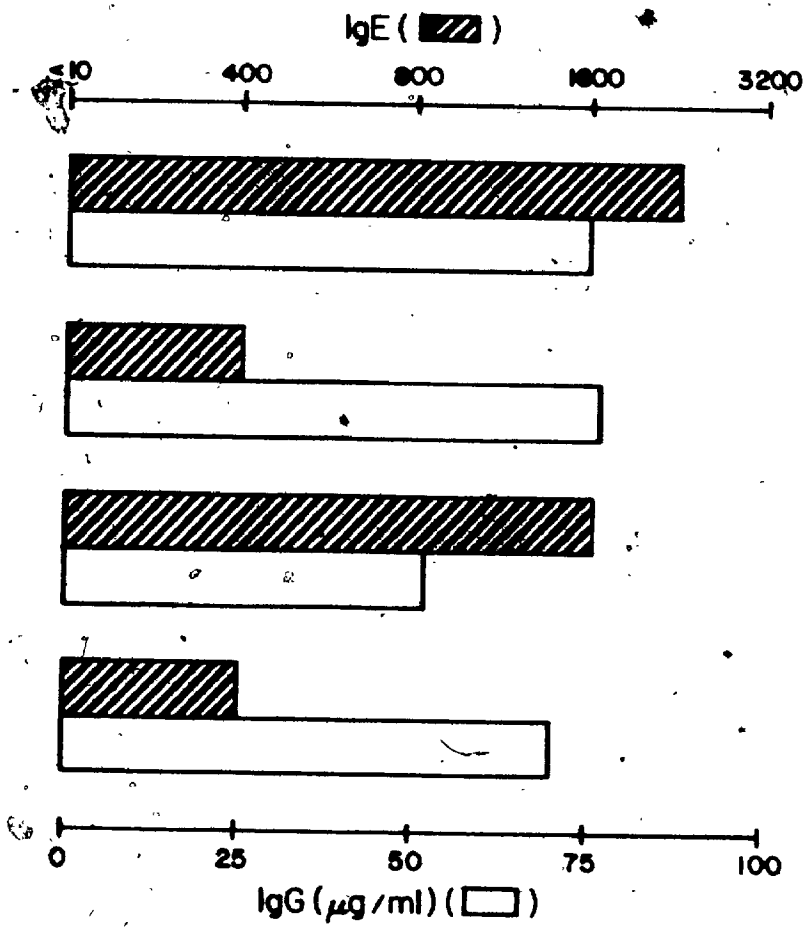


FIGURE 9: Abrogation of ongoing anti-OA IgE responses operates through T suppressor cells. Fifty million spleen cells from normal mice or from mice that were primed with 2 ug OA in alum and later injected with saline or OA-POL (see inset) were treated with monoclonal anti-Thy 1.2 antibody and complement, complement alone, or were left untreated. These were cotransferred to irradiated syngeneic recipients, along with 5×10^6 spleen cells from mice immunized 4 weeks earlier with 2 ug OA in alum. All recipients were boosted with 2 ug OA in alum and were bled 14 days later. Anti-OA IgE antibodies are given as PCA titers (■); IgG antibodies are expressed in ug/ml (□).

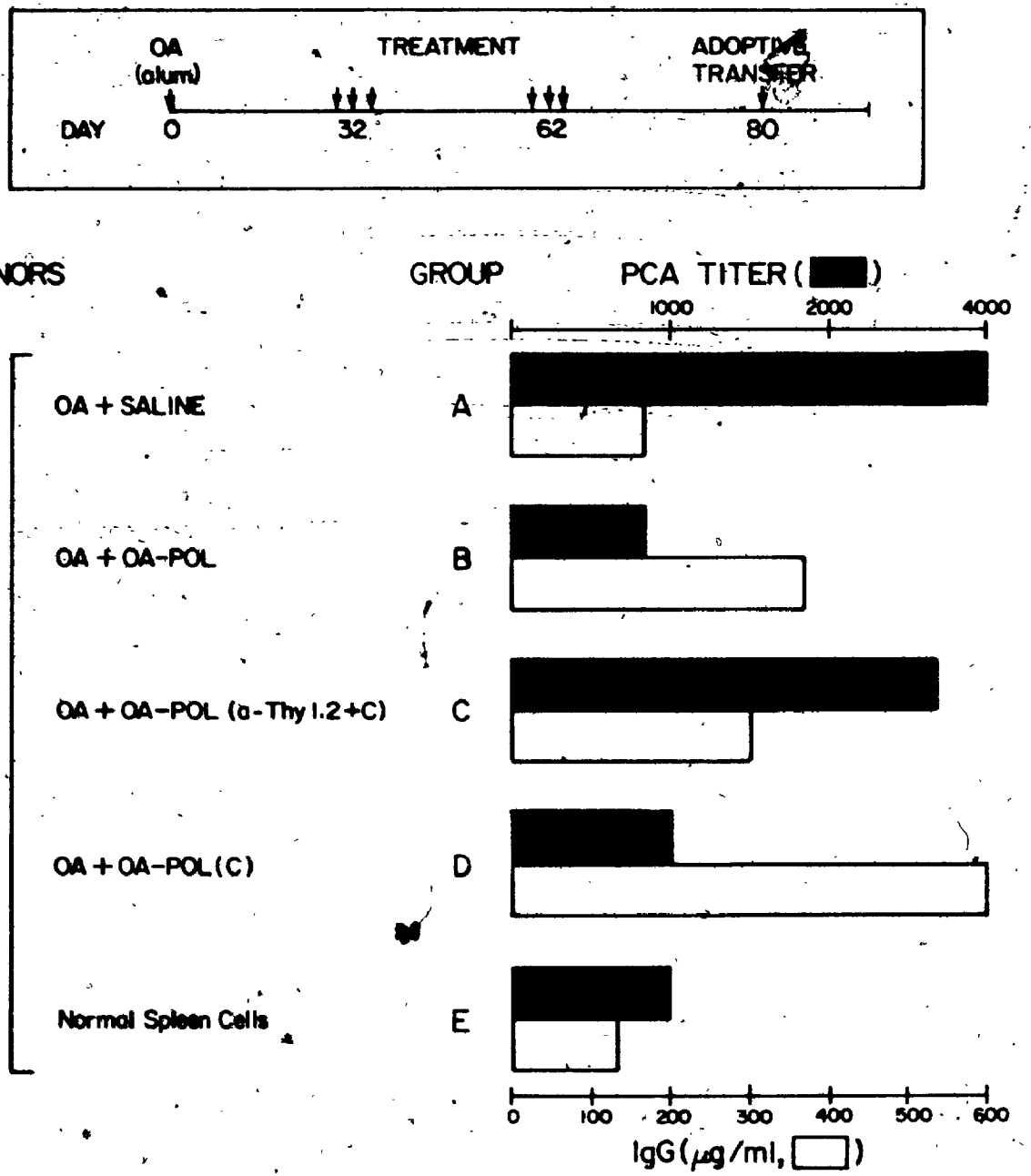
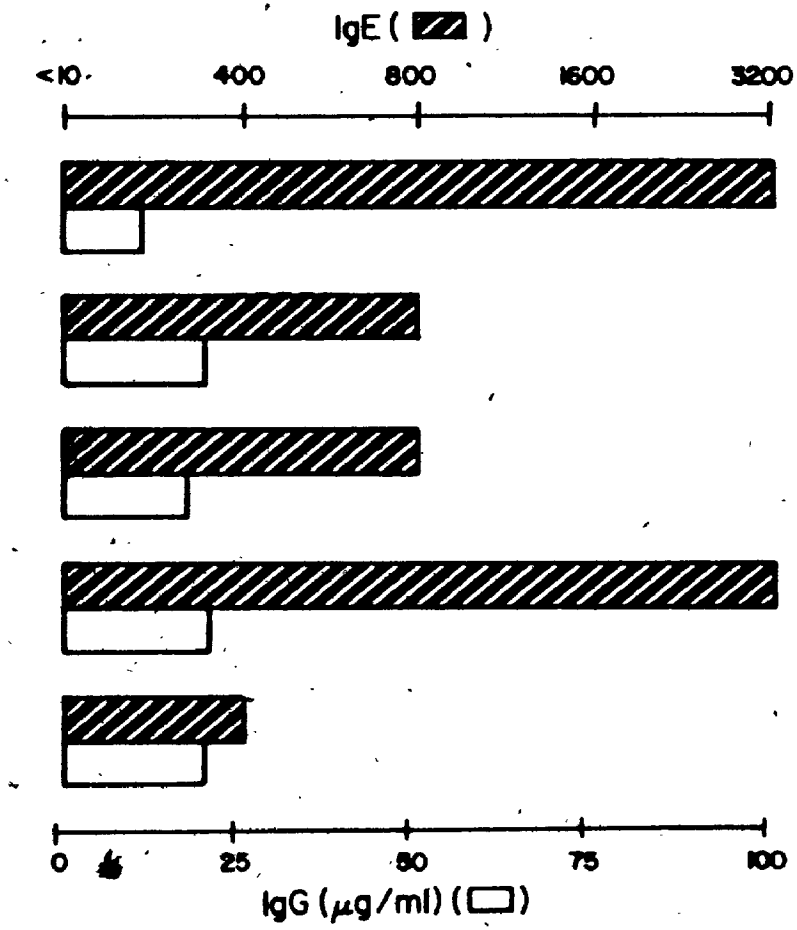

OA
(alum)
TREATMENT
ADOPTIVE
TRANSFER
DAY
0
32
62
80
DONORS
GROUP
PCA TITER ( )
1000
2000
4000
OA +
OA + SALINE
A
OA + OA-POL
B
OA + OA-POL (α-Thy 1.2+C)
C
OA + OA-POL (C)
D
Normal Spleen Cells
E
0
100
200
300
400
500
600
IgG (μg/ml,)

FIGURE 10: Demonstration by coadoptive transfer of Lyt 1.1 cell dependence of OA-POL-induced suppression. 5 x 10^7 spleen cells from OA-POL-injected donors were treated with monoclonal anti-Lyt 1.1 or anti-Lyt 2.1 antibody plus complement, with complement alone or were left untreated. These, or normal spleen cells, were mixed with 10^7 spleen cells from OA-primed donors and were injected iv into each of four X-irradiated (660 r) recipients per group. All recipients were boosted with 2 ug OA in alum on the day of transfer. IgE (▨) and IgG (□) anti-OA antibody levels of sera obtained 14 days later are shown.

Lyt 1+ DEPENDENCE OF SUPPRESSION

DONOR CELLS	
5×10^7	10^7
NORMAL	OA
OA-POL	OA
OA-POL (C)	OA
OA-POL (α-Lyt 1·1+C)	OA
OA-POL (α-Lyt 2·1+C)	OA



These results strongly suggest that a Lyt 1 T cell, but not an Lyt 2 T cell, must be transfered from OA-POL treated mice for successful induction of antigen- and isotype-specific suppression. Whether the Lyt 1+ cell is itself a suppressor-effector or a suppressor-inducer is discussed below.

IV. Long Term Immunoregulation of IgE Responses Following OA-POL Treatment:

Generation of memory T suppressor cells

The preceding experiments suggested that suppression of the IgE response following treatment with OA-POL was long-lasting since it was still in effect more than 250 days after the termination of treatment. In order to study its longevity and mechanism of action, a separate series of experiments was carried out in which groups of mice were pretreated with a single course of 3 OA-POL injections followed by a standard immunization with 2 ug DNP-OA (alum) given at various intervals after treatment. The mice were boosted with 2 ug DNP-OA (alum) 28 days later. The results presented in Figure 11 show that both anti-DNP and anti-OA PCA titers were drastically suppressed for a period of at least 7 months when compared with the saline-treated and age-matched controls. In fact, suppression was not only maintained over the seven month course of the experiment but gradually increased with time. These results, repeated in 3 separate experiments, raised the question as to whether OA-POL treatment could lead to the induction of long-lived memory suppressor cells selectively affecting the anti-OA IgE antibody response. Consistent with previous observations (Table V), pretreatment with native antigen under these conditions does not lead to supression of IgE responses.

TABLE X

Antigenic-specificity of long-lived suppression

TREATMENT[1]	IMMUNIZATION[2]	Anti-DNP PCA titer at day 14	21
OA-POL	DNP-OA	20	20
Saline	DNP-OA	800	1600
OA-POL	TNP-KLH	40	80
Saline	TNP-KLH	20	40
OA-POL	DNP-Asc	40	80
Saline	DNP-Asc	80	80

[1] Mice were treated with 80 ug OA-POL or with saline on days -200, -198, -96 and -14, -12, -10.

[2] Mice were immunized with 2 ug DNP-OA, 10 ug TNP-KLH or 10 ug DNP-Asc, each in alum, on day 0.

FIGURE 11: Demonstration of longlasting suppression following OA-POL pretreatment. Mice were injected with saline or with 80 µg OA-POL on days 0, 2 and 4. Beginning on day 35 (week 5) and at monthly intervals thereafter, one group of mice was primed, and boosted 28 days later with 2 ug DNP-OA (alum). IgE, expressed as PCA titer, was determined 14 days after the first DNP-OA injection (▨) and 7 days after booster (□). IgG is expressed as ug antibody/ml presented in round brackets.

TREATMENT	TREATMENT/CHALLENGE INTERVAL (weeks)	PCA TITER anti-DNP	PCA TITER anti-OA
SALINE	5	(21) / (141)	(<5) / (34)
OA-POL	5	(<5) / (150)	(200) / (246)
OA-POL	9	(<5) / (110)	(168) / (171)
OA-POL	13	(<5) / (240)	(171) / (190)
OA-POL	17	(<5) / (124)	(75) / (57)
OA-POL	23	(<5) / (151)	(72) / (23)
SALINE	23	(<5) / (172)	(<5) / (50)

Antigenic specificity of long-lived suppression

Suppression induced by conventional OA-POL pretreatment (i.e. day -16,-14,-12) leads to antigen-specific suppression of the IgE response. Data presented in Table X demonstrates that the long-lived supression shown above is also antigen-specific.

Demonstration of memory T suppressor cells

Antigen-primed donor mice were prepared by a single injection of 2 ug OA (alum) 30 days before transfer. OA-POL treated cell donors were prepared in the usual fashion by three 80 ug injections (i.e. days -16,-14,-12). Putative memory suppressor cells were generated by a course of three 80 ug OA-POL injections on days -200,-198,-196. Two courses of OA-POL treatment, beginning on day -200 and again beginning on day -16, were given to a third group of cell donors. Spleen cells from OA (alum) primed donors were transferred to irradiated recipients alone or in combination with cells from OA-POL treated donors. Recipients were challenged the same day with 2 ug OA (alum). IgE and IgG antibody levels were determined on sera collected 7, 14 and 21 days later. The results for days 7 and 14 are presented in Figure 12.

Dose response experiments (not shown) have established that adoptive transfer of 15×10^6 spleen cells from OA-POL pretreated mice (standard course: day -16,-14,-12) along with 10^7 OA (alum)-primed cells gave too low a ratio of suppressor to OA-primed cells for effective suppression of IgE responses. In contrast, Figure 12 demonstrates that even under such limiting conditions (15×10^6 OA-POL cells), cotransfer of OA-POL-boosted memory suppressor cells led to 96% suppression of day 14 anti-OA PCA titers. It can be seen that the transfer of 35×10^6 cells from any of

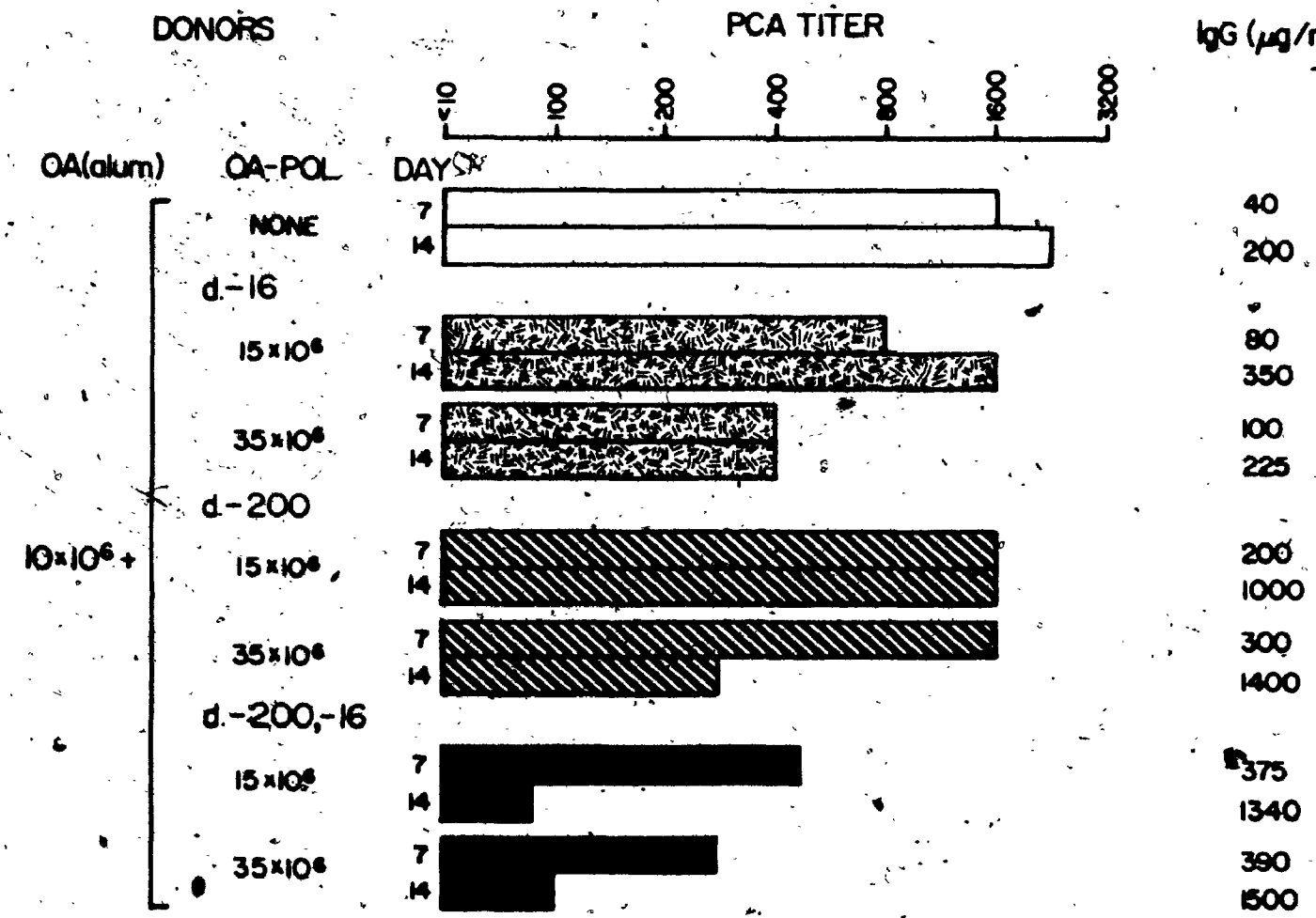
the three OA-POL treated donor populations suppressed the IgE response of 10^6 OA-primed cells to a significant degree without concomitant suppression of the IgG response (day 14 PCA titers of 400, 320, and 100 vs. 2400). Again, suppression was most striking in the group receiving cells from OA-POL boosted donors.

In addition to displaying more potent suppressive effiency and being effective at lower cell concentrations, OA-POL boosted memory suppressor cells also display more rapid induction of suppressive activity. Suppression of IgE responses following transfer of 15 or 35 x 10^6 cell occurs by day 7 after transfer only in the boosted memory suppressor cell group. In contrast, 14 days (and 35 x 10^6 cells) are usually required for suppression in the groups receiving conventional Ts or unboosted memory suppressor cells.

Anti-OA IgG antibody levels were either unchanged or, in the case of recipients of cells from day -200 and day -200; -16 OA-POL treated donors, elevated. The results obtained on day 21 (not shown) were essentially the same as those of day 14. It can be seen therefore that OA-POL treatment induces not only suppressor cells transferrable under conventional conditions but also a long-lived ($\geq$200 days) population of memory suppressor cells that is boostable by either OA-POL or by native OA itself.

FIGURE 12: Suppressor cell memory demonstrated by adoptive transfer. OA (alum) primed donors were immunized 28 days prior to transfer. Different OA-POL cell donor populations were given 80 ug injections on three alternate days beginning at times shown. Cells were mixed as indicated and transferred to irradiated recipients that were boosted the same day with 2 ug OA in alum. Serum antibody levels 7 and 14 days after transfer are shown.

SUPPRESSOR CELL MEMORY DEMONSTRATED BY ADOPTIVE TRANSFER



DISCUSSION

This investigation demonstrated that treatment of CBA mice with GA-polymerized ovalbumin (OA-POL) leads to highly efficient antigen-specific suppression of primary and secondary IgE responses. On the other hand, IgG antibody and delayed hypersensitivity responses were not suppressed.

Previous studies in rats, guinea pigs and man have established that GA-polymerized proteins display a marked reduction in allergenicity and antigenicity compared to the unmodified allergen. For this discussion allergenicity is defined as the capacity of the protein to react with specific IgE (anti-OA) and antigenicity is defined as the capacity to react with IgG.

Human immunotherapy with GA-polymerized allergens has been shown to result in decreased IgE levels and an improvement in clinical symptoms to an extent at least as great as that obtained following hyposensitization with native allergens. Several hypotheses have been advanced to account for the clinical efficiency of GA-polymerized allergens. Patterson (1981) feels that the retained capacity of these preparations to induce "blocking" IgG antibodies is responsible for their suppressive activity. No published attempt has been made to examine altered T cell reactivity in patients receiving GA-polymerized antigens despite the clear indication (Rocklin et al, 1980) of antigen-specific suppressor cell generation in patients under other forms of immunotherapy.

In view of the fact that clinical trials with a variety of other chemically modified allergens (UD-AgE, MPEG-AgE, AgE: D-GL) have proven unsuccessful (Norman, 1982) while glutaraldehyde-modified antigens are extremely promising, it is of primary importance to determine the

mechanism by which these polymers act.

Most early studies of the immunologic properties of GA-modified proteins were carried out with crude, highly heterogeneous preparations of Timothy grass pollen extracts (Moran et al, 1976) OA-BSA copolymers (Habeeb, 1969) or ragweed pollen extracts (Patterson et al, 1973). For the present study, it was felt that unequivocal characterization of GA-modified proteins (with respect to molecular weights, antigenicity, allergenicity, immunogenicity, suppressive efficiency, determination of which chemical modifications are necessary for suppression) could be best achieved with a defined range of GA-modified ovalbumin preparations, each with different molecular parameters. The production and isolation of such preparations of restricted heterogeneity made it possible to associate specified molecular characteristics of GA-modifed OA with the capacity to induce supression of IgE responses.

Treatment of protein with GA usually leads to polymerization by the crosslinking of amino acids, predominantly lysine, at their epsilon-amino groups. The extent of GA substitution, ratio of inter -to intra-molecular bonding and ultimately the size of the polymers formed, depends greatly upon the reaction conditions selected. Thus, adjustment of pH, protein concentration, molar ratios of the reactants and reaction times made it possible to produce lightly (OA_1-L) or heavily (OA_1-H) glutaraldehyde--substituted ovalbumin preparations. These fractions, as collected from gel filtration media for experimental purposes, were considered monomeric because they were obtained at the same elution volume as the major peak for native ovalbumin (40,000 d.) Ovalbumin polymers of low (160,000-200,000 d., OA_4), moderate (2-10 x 10^6d, OA_{175}) and high (35 x 10^6d, OA-POL) molecular weight were also generated.

It is conceivable that even under strictly controlled conditions, the preparations obtained are not homogeneous and probably vary somewhat from batch to batch. However the results reported here were obtained using many GA-modified OA batches over a period of three years. Before use, each batch was purified by gel filtration and only the fraction corresponding to the appropriate molecular weight range was used. While it is difficult to determine the extent of variation in the physicochemical spectrum among the isolated preparations, it must be noted that both the elution profiles and the immunologic properties of all batches of any given preparation were practically identical.

Antigenic crossreactivity between GA-modified OA and native OA, as measured with IgE anti-OA or IgG anti-OA antibodies, gave very similar results (Figures 5,6). OA_1-L functionally lost about 40% of the antigenic determinants of native OA. Increasing the degree of chemical modification (OA_1-H) resulted in a further decrease in antigenicity (82%) and allergenicity (75%), still in the absence of polymerization. Increasing molecular weight from 40,000 d (OA_1-H) to 35,000,000 d (OA-POL) yielded only a 7-18% additional decrease in antigenic crossreactivities. Clearly, the decrease in antigenicity (89%) and allergenicity (92%) of OA-POL is predominantly a consequence of chemical alterations of ovalbumin determinants rather than polymerization and increased molecular weight.

The decreased crossreactivity of OA-POL with OA at the anti-OA IgE and IgG antibody level is not reflected as a similar decrease in reactivity at the T cell level. Table II demonstrates that mice primed for delayed hypersensitivity with OA display significant footpad swelling when challenged 13 days later with OA. DTH challenge of identically

primed animals with OA-POL shows that it is at least as efficient in eliciting DTH reactions as is the native antigen. Thus OA-POL, while losing most of its capacity to react with B cells specific for OA, has not lost the ability to react with OA specific T_{DTH} cells.

The question of whether the increased ability of OA-POL to elicit anti-OA DTH reactions (Table II: T_{24}/T_0 of 1.33 vs. 1.16) is attributable to an increased availability/improved presentation of T cell-reactive determinants, or merely to a slower rate of diffusion of this high molecular weight molecule from the footpad, was not pursued as it was beyond the scope of this study.

We felt that any alterations in immunogenicity that might occur as a consequence of GA-modification would be of considerable importance. Clinically, nearly every ragweed sensitive human responds to AgE (Norman et al, 1980). Although many patients are sensitive to one or more "minor antigens" (Ra5, Ra3) in addition to AgE, the pattern of sensitivities varies widely between individuals. Commercially available hyposensitization preparations contain a broad mixture of related allergens so it is conceivable that injection of extraneous minor allergens might lead to the acquisition of new sensitivities by the patient receiving therapy.

A normal, non-atopic individual volunteered for immunization with a grass pollen extract to determine the antigenicity and immunogenicity for "blocking" IgG antibodies of this extract (Turkeltaub et al, 1978). Initially the individual had negative skin tests to grass pollen extract and no known allergic symptoms. After injection of the extract, the volunteer developed IgE to grass pollen and now has grass pollen symptoms at the same time that naturally allergic patients do.

As it is not always feasible to determine the presence/absence of sensitivity to each minor allergen and because pure preparations of each individual allergen are not usually available, the potential immunogenicity (for IgE) of any treatment preparation must be of great interest. It was decided here to determine if the decreased antigenicity and allergenicity of OA-POL is associated with a similarly decreased immunogenicity for IgE responses.

If GA-modification of proteins results in decreased immunogenicity for IgE yet retains the capacity of the relevant proteins to induce suppression of ongoing responses, then the risk of introducing new sensitivities would be minimized while the patient was deriving the benefits of treatment of his original sensitivity.

In our experiments, multiple injections of OA-POL in saline never result in the induction of IgE responses (data not shown).

To further test the potential immunogenicity of GA-polymerized OA for IgE antibody induction, OA or OA-POL were injected in alum, conditions optimal for the induction of IgE. The finding that OA (alum) immunization resulted in a mean PCA titer of 389 while immunization with OA-POL (alum) yielded primary PCA titers almost 15-fold lower suggests an added advantage of hyposensitization with GA-modified protein antigens. Inadvertent sensitization of atopics with extraneous allergens to which they were previously tolerant may be less likely when these preparations of decreased IgE immunogenicity are used for immunotherapy.

The purification of several GA-modified OA preparations and the quantification of their decreased allergenic, antigenic and immunogenic potential provided a solid basis and justified the extension of these studies into the area of <u>in vivo</u> IgE immunoregulation.

After establishing that treatment with OA-POL was capable of interfering in a dose-dependent fashion with the induction of IgE responses in naive mice (Table IV), the molecular characteristics of OA-POL relevant to establishment of the suppressed state were investigated. Previous studies which produced conflicting conclusions as to the immunologic effects of GA-polymerization (Attallah et al, 1975; Wheeler et al, 1976) may have been influenced by the heterogeneity of the preparations used. Such discrepancies may be easily reconciled by the notion that the wide range of products generated by GA-polymerization may, under certain circumstances, contain a restricted spectrum of molecules displaying only one of two opposing immunologic properties (induction vs. suppression). It was possible that the suppression of IgE responsiveness induced with OA-POL might be attributed to the polymeric status of the molecule rather than to intrinsic chemical modifications also present on modified, unpolymerized molecules. Thus, the question arose as to whether antigen modification with glutaraldehyde under conditions not conducive to polymerization would still yield suppressive molecules.

It was found that suppression of IgE responses occurs only following treatment with high molecular weight polymerized preparations (Table V). Chemically modified, non-polymeric preparations (OA_1-L, OA_1-H) and polymers of low molecular weight (OA_4) were not effective. This, and the correlation between increasing molecular weight and increasing suppressive efficiency (OA_{175} vs. OA-POL), supports the contention that polymerization, rather than other chemical modifications, is the crucial characteristic for the establishment of suppression. This is in marked contrast to the results discussed above in which decreased antigenicity and allergenicity are primarily attributable to non-polymeric chemical

modification.

It should, however, be noted that because suppression requires polymerization that this is not to say that other chemical modifications may not contribute to the suppressive efficiency of these polymers. Whether polymerization of OA could be achieved without other chemical modifications by the use of another crosslinking reagent and if such polymers would have suppressive activity is beyond the scope of the present investigation.

Treatment of mice with well established IgE responses, a closer parallel to the human atopic situation than treatment of naive animals, has been studied in several systems (Takatsu et al, 1975; Lee et al, 1978; Katz, 1980). As one might expect, shifting the immunological balance from antibody production to suppression is considerably more difficult to achieve when dealing with an expanded, antigen-primed lymphocyte population than in the naive animal. Nevertheless, suppression of IgE responsiveness under such conditions is clearly a necessary prerequisite for any useful preparation.

In the present study, abrogation of the capacity of primed mice to generate secondary IgE responses was not achieved following one course of treatment with OA-POL. This is in spite of 25-100 fold increases anti-OA IgG ("blocking") antibody levels.

Treatment with two courses of OA-POL (Figure 7) resulted in 8-10 fold suppression of IgE anti-OA responses over a period of greater than 300 days. Anti-OA IgG levels following two courses of OA-POL treatment were no different than those after one course of OA-POL. The results indicate that anti-OA IgG produced following OA-POL treatment is not directly relevant to the suppression of IgE responses in this system.

Further evidence supporting such an assertion is the observation that treatment with unmodified Ovalbumin 30, 32, 34 and 60, 62, 64 days after sensitization also induces a high level of anti-OA IgG which was maintained for the duration of the experiment. The IgE response was suppressed only to day 161.

OA-POL-induced suppression was shown to be both antigen-specific and IgE class-specific as neither IgG antibody nor delayed hypersensitivity responses were suppressed. In this respect OA-POL is distinct from a wide variety of other chemically modified antigens (UD-OA, OA-PEG, DNP-PVA, DNP-D:GL) with which suppression was not restricted to the IgE class. Confining the immunosuppressive effects of hyposensitization to the targeted immunologic parameter (allergen-specific IgE) rather than establishing a pan-specific state of suppression would seem to be a desirable goal. The mechanism by which OA-POL treatment establishes such a state of suppression became the focus of the study.

Since the discovery that Ts cells play a central role in regulation of antibody synthesis, work in several laboratories has concentrated upon the function of these cells in IgE antibody production. Essentially, three distinct patterns of T cell-mediated suppression appear to operate in the mouse. One is antigen-specific and controls antibody responses of more than one Ig class, i.e. IgE and IgG; the second is antigen-nonspecific but is selective for the IgE class while the third is both antigen- and isotype-specific.

Initial identification of antigen-nonspecific Ts cells in low IgE-responder mouse strains was immediately followed by reports that these cells were Lyt 1+ IgE class-specific (Watanate et al, 1977). Following stimulation of BALB/c mice with DNP-coupled mycobacteria, Suemura et al

(1977) described a class-specific Ts cell that modulates IgE but not IgG antibody responses. This suppression was hapten-specific for its induction but nonspecific in its effect, as it suppressed IgE responses to non-crossreactive antigens.

In other instances, antigen-specific Ts cells were not found to act in an Ig-non-selective fashion. Examples include the suppression induced following treatment with urea-denatured ovalbumin (Takatsu et al, 1975) and with protein-polyethylene glycol conjugates (Lee et al, 1978).

Earlier reports of both antigen- and IgE class-specific unresponsiveness led to the suggestion that T helper cells collaborating with IgE-forming B cells were different from those collaborating with IgG-B cells and that a selective lack of the former was responsible for IgE class-specific unresponsiveness (Kishimoto et al, 1973).

Recently, antigen-specific and IgE class-selective suppression were found associated, following administration of protein-pullulan (Usui et al, 1979) and of protein-fatty acid conjugates (Segawa et al, 1981) (Reviewed in detail at Introduction). The suppression described here falls in this last category. The factors determining which of the three distinct patterns of suppression mentioned above operate in any given situation are at present obscure, but the nature of the immunogenic stimulus seems toplay an essential role. Segawa et al (1981) suggested that the generation of IgE-selective Ts cells following treatment with fatty acid-substituted OA could be attributed to the hydrophobic character of the molecule which may not permit its effective processing and/or its presentation by macrophages to the T helper cells. The GA-polymerized ovalbumin molecule also appears to acquire increased hydrophobicity. We believe, however, that deficient macrophage processing and presentation

are unlikely for several reasons: (1) IgG antibodies, which also require effective antigen presentation, are produced in normal amounts (2) hydrophobic antigens have been shown in many instances to be better processed or more effectively incorporated into macrophage membranes than soluble antigens (Gallily et al, 1968: Yasuda et al, 1979), and (3) when OA-POL-treated mice were immunized with DNP-OA in alum, IgE anti-DNP levels detected 7 days after DNP-priming were always higher than in the nontreated controls while suppression appeared on day 14 (Table V). This clearly indicated that T helper cells were effectively produced. This helper effect that was readily abrogated may mean that treatment with OA-POL does not differ radically from treatment with native OA except insofar as shifting more effectively the balance between Th and Ts towards Ts cells. If this were the case, the difference between the two types of treatments would be quantitative rather than qualitative. While this in itself would still leave OA-POL treatment with a marked advantage over native OA, there is sufficient evidence to suggest that the two treatments are qualitatively different. First, both anti-hapten and anti-OA IgE antibodies were suppressed following OA-POL administration. In contrast, only anti-hapten IgE antibodies were decreased following native OA pretreatment. The apparent decrease in anti-DNP antibodies in this latter group may be due not to Ts cells but to the presence of an expanded population of anti-OA B cells successfully competing with a relatively small number of anti-DNP B cells for Th cells or for antigen.

The phenotype of Ts cells induced by OA-POL treatment differs from that conventionally reported in other murine systems, the majority of which are Lyt 1-,2+3+. It should be remembered however that these cells have antigenic-specificity or isotypic-specificity but not both.

Concurrent antigen-specific, isotype-specific T cell mediated suppression was reported only once before (Segawa et al, 1981) and the T cell phenotype was not determined. Suppression in this study is dependent upon Lyt 1, but not Lyt 2, cells from OA-POL animals. Whether this means that the suppressor-effector cell is Lyt 1+, 23- cell remains an open question. It is conceivable that the antigen- and isotype-specific suppression may be effected by a novel Lyt 1+, 23- suppressor cell. Alternatively an OA-POL induced Lyt 1+, 23- T cell may act as an inducer of suppression, triggering uncommitted Lyt 123 cells to differentiate into Lyt 23+ suppressor-effectors. The presence of an undepleted Lyt 123 cell population among the cotransferred OA (alum)-primed spleen cells (Figure 10) makes such a possibility feasible.

A separate question concerns the mechanism by which antigen-specific, isotype-specific, T cell mediated suppression acts. Whether such suppression reflects the existence of a single Ts cell with dual antigenic and isotypic specificity, the combined effect of an antigen- specific Ts cell and an IgE isotype-specific Ts cell (Sugimura et al, 1982) or different sensitivities of IgE-B and IgG-B cells to a common T suppressor mechanism (Katz et al, 1974) is unresolved. It is interesting to note however that Richman et al (1981) reported simultaneous induction of distinct antigen-specific IgG-specific Ts cells and antigen-specific, IgA-specific Th cells in murine Peyer's patches. Independently, Ts cells selective for IgG antibody responses have been described in picrylchloride contact sensitized mice that at the same time were displaying normal IgE responses to the TNP-hapten (Thomas et al, 1981). It would be difficult therefore to attribute isotype-specific suppression exclusively to different susceptibilities of B cells to suppressive signals.

The role of IgG anti-OA "blocking" antibody in this system must be addressed. As reviewed at the Introduction, one school of thought accepts that increases in allergen-specific IgG result in decreased IgE levels and clinical improvement. Another argues that such increases are of no predictive or causative value in human hyposensitization. The present results give clear evidence of T suppressor cells selectively affecting the anti-OA IgE response. Such cells are demonstrable under a wide variety of experimental conditions. The lack of an inverse correlation between anti-OA IgG and IgE levels makes it difficult to justify a simple cause and effect relationship for "blocking antibody". Furthermore the inability of serum from OA-POL treated animals to transfer suppression (data not shown), taken with the demonstrated ability of T cells from these animals to do so, argues strongly against a direct role for blocking antibody in this system.

Human hyposensitization studies with GA-ragweed support such logic. In a multicentre clinical trial, suppression of anti-ragweed IgE production was much greater after two years of treatment than one. No difference was found in the level of "blocking" IgG antibody from year to year, leading the authors to question the validity of its competitive inhibitory role in vivo (Knight et al, 1979).

Late ongoing murine IgE responses are said to be much less amenable to suppression than early ones (de Weck et al, 1982). In this study, OA-POL treated groups have been shown to be suppressed for at least 200-300 days. A single course of OA-POL pretreatment given 23 weeks prior to primary, 27 weeks prior to secondary DNP-OA (alum) immunization caused 80-90% suppression of IgE responses in three experiments. Similarly, a dual course of OA-POL injections abrogated ongoing IgE responses for a

period of at least 321 days in spite of six additional OA (alum) booster injections. This experiment was also performed three times. While the absolute titers of animals treated with OA-POL after OA (alum) sensitization (Figure 7) are higher than those of animals treated prior to sensitization (Table V), the magnitude of suppression resulting from OA-POL treatment is equally good under either set of circumstances.

Such suppression is not attributable to general senescence of the immune system since parallel, saline treated control groups responded in a normal fashion with both IgE and IgG antibodies. Independent studies also indicate that CBA, and a variety of other inbred mouse strains, have unchanged capacity to generate antibody responses at 2 years of age.

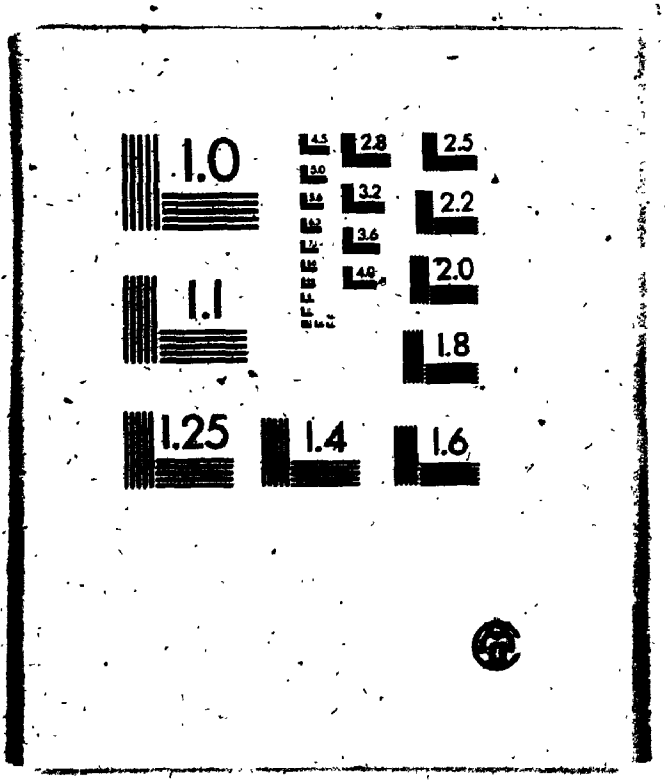
The mechanism by which long term tolerance is maintained remains controversial. Although most murine Ts are relatively short-lived (30-40 d.), unresponsiveness in the animal may last for greater than 150 d. after a single i.v. injection of deaggregated protein (HGG)(Doyle et al,1976). It has been argued that this apparent discrepancy indicates that longterm tolerance may be independent of Ts cells, the presence of which could be a regulatory epiphenomenon of little or no relevance to the mechanism of unresponsiveness.

The experiments presented here indicate that memory Ts cells (Tsm) are represented in murine spleens and can be recalled after extended periods of time. These data and those of Loblay et al, (1978, 1983) suggest an alternate mechanism by which longterm tolerance may be maintained.

It can be seen in Figure 12 that memory Ts cells are recallable not only by OA-POL booster but also by booster with the sensitizing antigen, native OA. The potential therapeutic advantages of inducing Ts cells with

GA-polymerized antigen and having their activity boosted by later exposure to the unmodified sensitizing allergen are obvious. From a theoretical perspective, such a finding suggests the possibility that "suppressive determinants" common to the two forms OA and OA-POL, may be responsible for the preferential stimulation and recall of Ts cells. Previous evidence suggests that an epitope on a protein antigen may be presented to, and may result in the stimulation of, either Th or Ts cells but not both (Adorini et al, 1979; Krzych et al, 1982). Distinct peptides of the same molecule were responsible for immunogenicity and for generating Ts cells (Swanborg, 1975; Sercarz et al, 1978). Indeed, instances exist where Th and B cells were directed against different regions of the same antigen molecule (Senyk et al, 1971; Maizels et al, 1980). Glutaraldehyde modification of ovalbumin may result in preferential presentation of Ts-triggering determinants that lead to the expansion of these clones and the generation of memory Ts cells.

One question that arises from the present experiments is whether the long-lived Ts cells are themselves Ts effector cells or represent antigen-primed precursors endowed with immunological memory that differentiate into Ts effector cells upon restimulation with antigen. The delayed onset of suppression observed in recipients of 35 x 10^6 cells from donors treated with OA-POL on day - 200 only, (Figure 12) suggests that differentiation from an antigen-primed Ts precursor to a Ts effector is mandatory for suppression to be detected. Had these cells been fully differentiated long-lived Ts effectors, suppression should have been evident immediately after transfer, as seen on day 7 in the groups receiving 35 X 10^6 cells from donors treated on day - 16 (Ts and OA-POL-boosted Tsm). Tsm display other classical attributes of



immunological memory as defined by others (Loblay et al, 1978; Liew et al, 1980), such as increased suppressive activity at a constant number of cells and the requirement for fewer cells. In figure 12, a comparison of suppression following transfer of constant numbers of cells shows that recipients of OA-POL day -200; day -16 are significantly better suppressed than recipients of the same number of cells from either day -16 or day -200 OA-POL treated donors. The necessity for fewer cells is illustrated by the fact that 15 X 10^6 of the OA-POL boosted Tsm population mediate better suppression than twice as many of the other OA-POL populations (day 14 PCA titers of 80 vs 400, 320).

The novel findings of this study can be summarized as follows. GA-modification of protein antigens can lead to the generation of a wide spectrum of modified molecules, all of which display significantly decreased antigenicity. However, only the high molecular weight ovalbumin polymers are capable of establishing potent suppression of IgE responses. This antigen- and isotype-specific suppression decreases IgE responses of both naive and previously sensitized mice by 80-90%. OA-POL induced suppression is Lyt 1+, T cell depehdent. In contrast with previous findings, the suppressed state was maintained for extended periods of time, even in the absence of further treatment. Long-lived suppressor cells are boosterable, even after more than 200 days, with OA- POL or native OA.

The study suggests that homeostasis of IgE responses may be maintained, at least in part, by long-lived and boosterable, antigen-specific, isotype-selective suppressor T cells. The experimental manipulation of such cells indicates that increasing their activity may provide a useful approach for the selective regulation of IgE-mediated hypersensitivity.

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