

# Modulation of the immune response to ragweed allergens by peptic fragments

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## Summary

Fragments of short ragweed extract were prepared by peptic digestion and fractionated by molecular exclusion chromatography. We focused on fragmenting a ragweed fraction that was enriched for *Amb a1*. Digestion products in the molecular weight range of 5–15 kD (fSRW) were examined for their antigenic, immunogenic and immunosuppressive properties. fSRW was poorly immunogenic and unable to induce ragweed-specific PCA reactions in rat skin or to bind to anti-ragweed IgG antibodies. Intravenous administration of fSRW either prior to or after intraperitoneal immunization with a ragweed preparation resulted in a significant suppression of the immune response. T cells isolated from lymph nodes of mice immunized with ragweed were stimulated by fSRW in a lymphoproliferation assay. Moreover, T cells from mice injected with fSRW were immunosuppressive when transferred into mice immunized with intact ragweed antigens. Our data demonstrate that peptic fragments (fSRW) of ragweed lack B-cell determinants while retaining their T-cell immunoregulatory properties. Potentially these peptides are less likely to cause adverse reactions in allergen sensitive patients and thus offer a new approach to allergen immunotherapy.

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## Introduction

Ragweed hay fever is caused by immediate hypersensitivity to allergens present in the pollen of the short ragweed plant. Immunotherapy for this disorder consists of subcutaneous injections of increasing doses of the short ragweed pollen extract. However, successful treatment with commercial ragweed preparations usually involves several years of therapy. To improve efficacy and shorten the time of therapy, a number of modified allergens have been developed and tested. Patterson *et al.* polymerized ragweed extract with glutaraldehyde [1]; Katz *et al.* prepared a conjugate of ragweed allergen with D-glutamic acid and D-lysine [2]; Schon and Lee used a conjugate of polyethylene glycol polymer [3]. Modification of the antigenic structure has been attempted by Marsh *et al.* who used formaldehyde treatment [4]; and Ishizaka *et al.* who utilized antigen denaturation [5]. None of these preparations have totally achieved the desired goals or

have been approved for use in the United States. We have recently shown that it is possible to suppress the immune response in mice to protein antigens such as bovine serum and honey bee venom phospholipase A2 by treatment with fragments derived by enzymatic digestion [6,7]. In this study, we extended our observations to allergenic proteins of short ragweed pollen, fragmenting them by peptic digestion. These ragweed fragments were characterized with respect to their immunogenicity, retention of antigenic structure, and the ability to suppress the primary and ongoing immune responses in mice.

## Materials and methods

### Mice

The experimental procedure adhered to the published *Guide for the Care and Use of Laboratory Animals* formulated by the National Institute of Health, and those formulated by the American Association for Accreditation for Laboratory Animal Care. Specific protocols were approved by the Committee on Animal Care of the University of Cincinnati.

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BDF<sub>1</sub> female mice, 9–12 weeks old were purchased from Jackson Laboratory, Bar Harbor, Maine. Each experimental group consisted of five animals and each experiment was repeated three times.

### Reagents

Defatted ragweed was kindly donated by Hollister-Stier (Spokane, U.S.A.) Sephadex was purchased from Pharmacia Corp. (Piscataway, NJ, U.S.A.). Ovalbumin, chymotrypsin, lysozyme and pepsin were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.).

### Preparation of ragweed fragments

One hundred grams of defatted ragweed was dissolved in 1 litre of distilled water and stirred overnight at 4°C. The water extract was centrifuged at 3300 × *g* (4500 r.p.m.) for 30 min at 4°C and the supernatant was filtered through Whatman filter paper (No. 1). Solid ammonium sulphate was added gradually with constant stirring over 4 hr at 4°C to produce a final concentration of 90%. The solution was left overnight in the cold then centrifuged at 3300 × *g* (4500 r.p.m.) for 30 min at 4°C. The precipitate was dissolved in a minimal amount of distilled water and desalted by passing over a Sephadex G25 column. This preparation, Fraction A, was lyophilized and stored at –70°C. To prepare an Antigen E (*Amb a I*) enriched fraction, further separation was done on a Sephadex G-50 (SF) column (2 × 90 cm) (Pharmacia) calibrated with ovalbumin, chymotrypsinogen and lysozyme as molecular weight markers: 3-ml aliquots were eluted and collected at a flow rate of 22 ml/hr using 0.1 N ammonium acetate buffer pH 6.9. The fraction of molecular size 20 000–35 000 D (Pool 2) was dialysed and lyophilized. Most of the *Amb a I* activity was found in Pool 2. Pool 2 was used in preparing antigenic fragments.

Digestion of Pool 2 was initiated by adjusting saline solubilized Pool 2 to pH 2.8 and by adding 0.007 mg pepsin/mg ragweed protein. The reaction was allowed to proceed for 4 hr at room temperature. The digestion was stopped by adjusting the mixture to pH 8.0 with 15 N NH<sub>4</sub>OH.

The pepsin digest of Pool 2 was fractionated on Sephadex G-50 superfine column. The major fraction (fSRW) consisted of digestion products having molecular weight in range of 5–15 kD.

Determination of *Amb a I* content was done using the potency test described in section 680.4 of Title 21 of the Food and Drug Administration (FDA) by a radial diffusion assay utilizing anti-*Amb a I* antiserum provided by FDA.

### Passive cutaneous anaphylaxis (PCA)

Doubling dilutions of mouse sera were injected intradermally into the shaven skin of anaesthetized Sprague-Dawley adult male rats. After 24 hr, 1 mg to 10 µg of a short ragweed preparation (either fSRW, Pool 2, or Fraction A) were added to 0.5% Evan's blue solution and injected through the penile vein of the rat. The reaction sites were observed after 20 min on the skin. The titre was expressed as the reciprocal value of the highest dilution giving a positive reaction of at least 5 mm in diameter. Each serum was tested in three rats.

### T cell proliferation assay

Mice were primed with the appropriate antigen in CFA, killed on day 9 and their popliteal and inguinal lymph nodes removed. Lymph nodes were ground into a single cell suspension. The T-cell population was enriched by passage over a nylon wool column and T cells (4 × 10<sup>5</sup> cells) were added to each well of a 96-well, flat-bottomed, tissue culture plate. Spleen cells from naive mice were used as antigen-presenting cells (APC). The APC were pulsed for 6 hr at 37°C and 5% CO<sub>2</sub> with the appropriate antigen before being added to T-cell suspensions. Tissue culture plates were incubated at 37°C with 5% CO<sub>2</sub> for 48–72 hr when 2 µCi of [3H]thymidine was added to each well. These plates were re-incubated for an additional 18–20 hr and then harvested with a Skatron cell harvester. Thymidine incorporation was measured by liquid scintillation counting.

### Antibody and antigen determination by ELISA

A quantitative ELISA technique was used as described previously [7]. Standard curves with an affinity-purified mouse IgG preparation were run each time when an assay was performed. The ELISA results are expressed in EU units with 1 EU equivalent to 1 µg of the standard. In the inhibition assays we employed several concentrations of antigens. The antigen dilutions were incubated overnight at 4°C with a constant dilution of antiserum. The mixtures were then assayed on ELISA plates coated with Pool 2. Pooled sera were collected from groups of five mice and each experiment was repeated at least two times. Significance of results was determined by Student's *t*-test.

### Pre-treatment and immunization of mice

Ragweed preparations were dissolved in saline and administered either intravenously or adsorbed on alum when injected intraperitoneally.

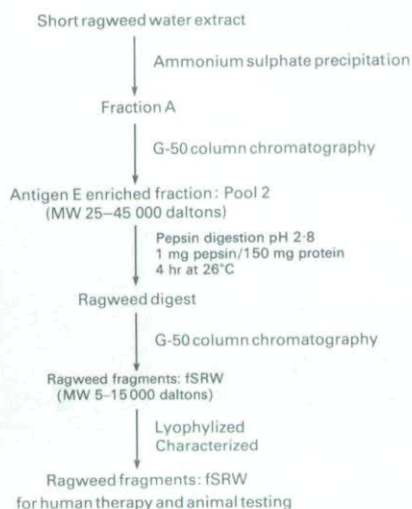


Fig. 1. Flow scheme of steps taken in preparation of ragweed fragments (fSRW).

#### Protein determination

The amount of protein by dry weight was determined using the Biorad Protein Assay (Biorad, Richmond, CA, U.S.A.) and by monitoring the absorbance at 280 nm.

#### Results

##### Preparation of Pool 2 and fSRW

Figure 1 is a flow scheme of steps taken to prepare Fraction A, Pool 2 and fSRW. Fraction A contained 5%

*Amb a I*. The second peak of the G50 sephadex column, Pool 2, was selected as the source for ragweed allergen in this study as *Amb a I* was most highly concentrated here (26% *Amb a I*). Pool 2 was then digested with pepsin and the digestion products were also chromatographed on Sephadex G-50. The second peak of this separation which contained peptides having molecular weight less than 15 kD served as the source of ragweed protein digest (fSRW) used in this study. The *Amb a I* antigen in fSRW was not detectable as fSRW would not precipitate in agar when reacted with anti-*Amb a I* antiserum.

##### Immunogenicity and antigenicity of Pool 2 and fSRW

Table 1 shows results of experiments in which the immunogenicity of the ragweed preparations was examined. BDF<sub>1</sub> mice were immunized i.p. with varying doses of Fraction A, Pool 2 and fSRW, with alum serving as adjuvant. The IgG response was measured by ELISA and the IgE response by PCA in rat skin. Mice responded well to i.p. immunization with Fraction A and Pool 2, but only very poorly to fSRW. This lack of immunogenicity of fSRW was further substantiated when fSRW was tested for binding to specific IgG and IgE anti-ragweed antibodies. Table 2 shows results of ELISA inhibition assays with fSRW, Pool 2 and Fraction A utilizing anti-Pool 2 mouse serum. As much as 1 mg of fSRW was unable to induce a PCA reaction in rat skin injected with murine serum containing ragweed IgE antibodies, nor did fSRW inhibit IgG antibody binding to ragweed antigen. Thus,

Table 1. Immunogenicity of ragweed preparations in BDF<sub>1</sub> mice

| Immunizing agent<br>injected i.p.<br>with 1 mg alum | $\mu\text{g}$ | ELISA IgG titre<br>(EU/ml)  |                             | IgE titre         |                   |
|-----------------------------------------------------|---------------|-----------------------------|-----------------------------|-------------------|-------------------|
|                                                     |               | 14 d                        | 28 d                        | 14 d              | 28 d              |
| Fraction A                                          | 100           | 720 $\pm$ 60 <sup>a</sup>   | 1260 $\pm$ 110 <sup>a</sup> | 640 <sup>c</sup>  | 320 <sup>c</sup>  |
| Fraction A                                          | 10            | 410 $\pm$ 35 <sup>a</sup>   | 770 $\pm$ 50 <sup>a</sup>   | 320 <sup>c</sup>  | 320 <sup>c</sup>  |
| Fraction A                                          | 1             | 78 $\pm$ 10                 | 46 $\pm$ 5                  | 80                | 40                |
| Pool 2                                              | 100           | 2320 $\pm$ 220 <sup>b</sup> | 3460 $\pm$ 280 <sup>b</sup> | 1280 <sup>d</sup> | 640 <sup>d</sup>  |
| Pool 2                                              | 10            | 860 $\pm$ 80 <sup>b</sup>   | 1220 $\pm$ 110 <sup>b</sup> | 1280 <sup>d</sup> | 1280 <sup>d</sup> |
| Pool 2                                              | 1             | 210 $\pm$ 15                | 180 $\pm$ 20                | 160               | 40                |
| fSRW                                                | 100           | 85 $\pm$ 10                 | 46 $\pm$ 5                  | 40                | 20                |
| fSRW                                                | 10            | < 5                         | < 5                         | 0                 | 0                 |
| fSRW                                                | 1             | < 5                         | < 5                         | 0                 | 0                 |

<sup>a</sup>  $P < 0.01$  for immune response to Fraction A compared with fSRW.

<sup>b</sup>  $P < 0.01$  for immune response to Pool 2 compared with fSRW.

<sup>c</sup>  $P < 0.05$  for immune response to Fraction A compared with fSRW.

<sup>d</sup>  $P < 0.05$  for immune response to Pool 2 compared with fSRW.

**Table 2.** Antigenicity of ragweed preparations

| Inhibitor tested | Minimal amount* needed to inhibit ELISA IgG ( $\mu\text{g/ml}$ ) | Minimal amount* needed to induce PCA reaction ( $\mu\text{g/ml}$ ) |
|------------------|------------------------------------------------------------------|--------------------------------------------------------------------|
| Fraction A       | 80                                                               | 40                                                                 |
| Pool 2           | 10                                                               | 10                                                                 |
| Fragments        | > 1000                                                           | > 1000                                                             |

\* Pool 2, mouse anti-Pool 2 antiserum.

enzymatic digestion of Pool 2 resulted in a preparation that contained only a limited number of B-cell determinants.

#### *Suppression of primary response by fSRW*

The loss of B-cell determinants in fSRW did not interfere with ability of fSRW to inhibit a primary immune response to Pool 2. Pre-treatment with fSRW or with Pool 2 intravenously suppressed the immune response to subsequent Pool 2 challenge (Table 3). This suppression included both IgG and IgE isotypic antibodies and was equally effective when induced by pre-treatment with proteins of Pool 2 or fSRW. These results suggest that regulatory T-cell determinants in fSRW capable of inducing immunosuppression remained intact.

#### *Suppression of ongoing IgE response*

BDF1 mice were immunized with Pool 2 in alum. On day 40, when mice still produced ragweed-specific IgE antibodies, half of the animals received six daily injections of

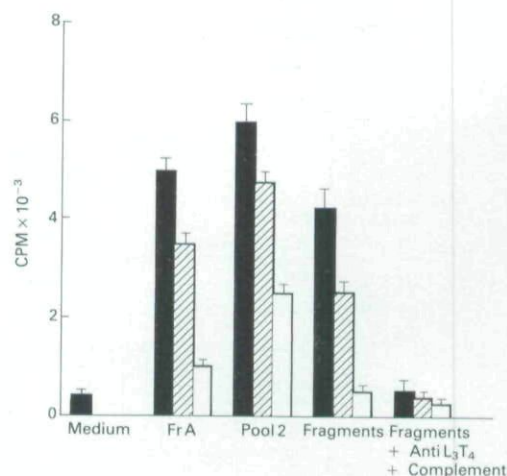


**Fig. 2.** Lymphocyte proliferation assay of cells isolated from lymph nodes of BDF<sub>1</sub> mice which were immunized with 100  $\mu\text{g}$  Pool 1 in CFA and stimulated in culture with Fraction A, Pool 2 or fSRW. (■) 100, (▨) 10 and (□) 1  $\mu\text{g}$ .

fSRW intravenously. Fifteen days later, IgE titres were reassessed. The mice which had received fSRW treatment showed a sharp decline in specific IgE antibodies as compared to the untreated mice (Table 4). This observation implies that an on-going IgE immune response can also be regulated by fSRW.

#### *T-cell determinants are preserved in fSRW*

To confirm that pepsin treatment of Pool 2 did not eliminate T-cell determinants, fSRW was tested in a lymphocyte proliferation assay. BDF<sub>1</sub> mice were immunized with 100  $\mu\text{g}$  Pool 2 in CFA and 9 days later

**Table 3.** Suppression of primary immune response to ragweed antigens by ragweed digest

| Pre-treatment<br>(days -9, -8,<br>-7) i.v. |     | Immunization<br>(day 0)<br>i.p. with alum |     | ELISA IgG titre<br>(EU/ml) |                       | IgE titre       |                 |
|--------------------------------------------|-----|-------------------------------------------|-----|----------------------------|-----------------------|-----------------|-----------------|
| $\mu\text{g}$                              |     | $\mu\text{g}$                             |     | 14 d                       | 28 d                  | 14 d            | 28 d            |
| Pool 2                                     | 100 | Pool 2                                    | 100 | 80 ± 10 <sup>a</sup>       | 80 ± 20 <sup>a</sup>  | 80 <sup>a</sup> | 20 <sup>a</sup> |
| Pool 2                                     | 100 | —                                         | —   | 20 ± 5                     | 10 ± 5                | 0               | 0               |
| —                                          | —   | Pool 2                                    | 100 | 1660 ± 175                 | 2200 ± 210            | 640             | 640             |
| fSRW                                       | 100 | Pool 2                                    | 100 | 120 ± 10 <sup>9</sup>      | 160 ± 75 <sup>a</sup> | 80 <sup>a</sup> | 80 <sup>a</sup> |
| fSRW                                       | 100 | —                                         | —   | 0                          | 0                     | 0               | 0               |

<sup>a</sup>  $P < 0.01$  for suppression of response to Pool 2 by i.v. pretreatment with fSRW or Pool 2 compared with control.

**Table 4.** Suppression of on-going IgE immune response to ragweed antigens

| Day 0              | Day 21    | Day 33    | Days 40-45      | Day 60    |
|--------------------|-----------|-----------|-----------------|-----------|
| Immunization with: | IgE titre | IgE titre | Treatment with: | IgE titre |
| Pool 2 100 µg      | 320       | 160       | fSRW 100 µg     | 20        |
| Pool 2 100 µg      | 320       | 160       | —               | 160       |

Day 0 immunization i.p. with 1 mg alum as adjuvant.

Days 40-45 treatment i.v. in saline.

T lymphocytes from the draining lymph nodes were removed and stimulated in cultures with Fraction A, Pool 2 and fSRW. All three preparations induced a similar degree of lymphocyte proliferation, indicating that fSRW retained T-cell determinants similar to Pool 2 and Fraction A (Fig. 2). The proliferating cells carried the L3T4 marker, as treatment with an anti-L3T4 antiserum and complement reduced T-cell proliferation to background levels.

#### *Suppression-induced fSRW is mediated by T cells*

Groups of mice were immunized with fSRW intravenously. Transfer of their splenic lymphocytes into

**Table 5.** Effect of T-cell transfer from mice treated with ragweed digest on the immune response to ragweed antigens

| T cells ( $1 \times 10^7$ )<br>transferred from<br>mice treated with | Immunization<br>with 10 µg<br>Pool 2 i.p.<br>in alum | ELISA IgG titre<br>(EU/ml) |                       |
|----------------------------------------------------------------------|------------------------------------------------------|----------------------------|-----------------------|
|                                                                      |                                                      | 14 d                       | 28 d                  |
| 3 daily i.v. inj.<br>100 µg fSRW                                     | +                                                    | 260 ± 40 <sup>a</sup>      | 120 ± 30 <sup>a</sup> |
| 3 daily i.v. inj.<br>100 µg fSRW                                     | —                                                    | < 10                       | < 10                  |
| 3 daily i.v. inj.<br>saline                                          | +                                                    | 1860 ± 90                  | 920 ± 30              |
| 3 daily i.v. inj.<br>saline                                          | —                                                    | < 10                       | < 10                  |
| 3 daily i.v. inj.<br>100 µg fSRW cells<br>treated with Thy 1.2       | +                                                    | 1450 ± 30                  | 870 ± 25              |
| No T cell transfer                                                   | +                                                    | 1220 ± 70                  | 750 ± 30              |

<sup>a</sup>  $P < 0.01$  for suppression of response to Pool 2 as compared with controls.

syngeneic naive mice ( $1 \times 10^7$  cells/mouse) prevented a primary immune response in the recipient to Pool 2 challenge (Table 5). This suppression was mediated by T cells, as shown by the removal of suppression following treatment of the transferred cells with anti-thy 1.2 antibody and complement. These data further support the conclusion that T-cell determinants in fSRW were preserved and remained functional.

#### **Discussion**

Pepsin digestion of *Amb a* I-enriched fraction of short ragweed resulted in generation of peptides that were poor immunogens. Also, these fragments reacted weakly with IgE and IgG anti-ragweed antibodies as documented by failure to induce a PCA reaction in rat skin and inhibit binding in an ELISA. Thus, we concluded that the enzymatic degradation caused destruction of major B cell determinants on the *Amb a* I molecule.

At the same time, these peptic fragments remained immunoregulatory and were capable of suppressing immune responses to ragweed antigens. This immunoregulatory function involved T cells since the fragments stimulated L<sub>3</sub>T<sub>4</sub>-positive, ragweed-specific T cells. Moreover, transferred T cells from mice treated with peptic fragments were immunosuppressive in naive mice challenged with short ragweed, indicating that the fragments were capable of stimulating T cells that regulate immunosuppression.

This study extends our earlier investigations on the modulation of immune response to other protein antigens in which we showed that antigenic fragments derived by proteolytic digestion of BSA and bee/venom phospholipase A<sub>2</sub> suppressed the immune response to the intact protein molecule [6,7]. Standring *et al.* recently reported that rye grass pollen extract digested by chymotrypsin produced fragments that did not react with either human or mouse IgG antibodies specific for rye grass pollen, but still retained the ability to react with antigen-specific T cells in a lymphoproliferation assay [8].

Peptides prepared by enzymatic digestion of a protein antigen comprise a mixture of heterogeneous molecules. Some of these peptides are immunoregulatory but so far it has not been possible to determine whether the same peptides can activate both T-helper and suppressor cells, or whether non-overlapping peptides are required for activation of functionally diverse populations of T cells. Clearly, in several experimental systems these peptide mixtures were found to be immunosuppressive [6,7]. The mechanism of immunosuppression remains a topic of lively controversy. While immunosuppression can be transferred by T cells to naive animals, as it has been done in our study, there may be different interpretations to

explain T-cell induced down regulation. Numerous investigators suggested existence of unique T-suppressor cells, but it is conceivable that unfavourable processing and presentation of the antigen to T-helper cells may result in induction of anergy. Our experimental design cannot exclude that possibility. It is intriguing to speculate whether there may exist a single region within a protein molecule that possesses all the necessary immunoregulatory properties. Available data utilizing well-defined peptides isolated from several protein antigens and tested in heterogenous human and animal populations indicate that a single peptide from a protein antigen does not seem to be adequate to activate the immune system in outbred populations, and that several T sites are involved in immunity initiated by T cells [9-11]. Thus, the polymorphism of peptide structures that is achieved by enzymatic digestion offers a distinct advantage for T-cell activation in heterogeneous populations. Degraded allergens, which lack reactivity with IgE antibody, should make ideal agents for immunotherapy as they have less potential to cause adverse reactions in sensitive patients.

## References

- 1 Patterson R, Suszko IM, Zeiss CR, Pruzansky JJ, Bacal E. Comparison of immune reactivity to polyvalent monomeric and polymeric ragweed antigens. *J Allergy Clin Immunol* 1978; 61:28-35.
- 2 Liu F, Katz DH. Immunological tolerance of allergenic protein determinants. A therapeutic approach for selective inhibition of IgE antibody production. *Proc Natl Acad Sci U.S.A.* 1989; 76:1430-4.
- 3 Schon AH, Lee WY. Suppression of immunoglobulin E antibodies with modified allergens. *J Allergy Clin Immunol* 1979; 18:705-22.
- 4 Marsh DG, Lichstein LM, Campbell DH. Studies on allergoids prepared from naturally occurring allergens. *Immunology* 1970; 18:705-22.
- 5 Ishizaka K, Kishimoto T, Delespesse G, King TP. Immunogenic properties of modified antigen E. *J Immunol* 1976; 113:70-7.
- 6 Michael JG, Pesce AJ, Litwin A, Freisheim J. Immunosuppressive properties of a phospholipase A2 antigen fragment. *J Allergy Clin Immunol* 1985; 75:200(abstr).
- 7 Ferguson TA, Peters T Jr, Reed R, Pesce AJ, Michael JG. Immunoregulatory properties of antigenic fragments from bovine serum albumin. *Cell Immunol* 1983; 78:1-12.
- 8 Strandberg R, Lavender EA, Wheeler AW, Spackman VM, Moran DM. Induction of T-helper cell activity by fragments of rye grass pollen extract produced by digestion with chymotrypsin. *Int Arch Allergy Appl Immunol* 1988; 87:337-41.
- 9 Berzofsky JA. Structural basis of antigen recognition by T-lymphocytes. Implication for vaccines. *J Clin Inv* 1988; 82:1811-17.
- 10 Berzofsky JA, Benussan A, Cease KB, Bouge JF, Cheymier R, Lurhuma Z, Salaun JJ, Gallo RC, Shearer GM, Zaguty D. Antigenic peptides recognized by T-lymphocytes from AIDS viral envelope-immune humans. *Nature* 1988; 334:706-8.
- 11 Lew AM, Langford CJ, Pye D, Edwards S, Corcoran L, Anders RF. Class II restriction in mice to the malaria candidate vaccine ring infected erythrocyte surface antigen (RESA) as synthetic peptides or as expressed in recombinant vaccine. *J Immunol* 1989; 142:4012-16.

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