ENHANCEMENT OF THE INTENSITY, PERSISTENCE, 
AND PASSIVE TRANSFER OF DELAYED-TYPE 
HYPERSENSITIVITY LESIONS BY PERTUSSIGEN IN MICE*

By W. A. SEWELL, J. J. MUNOZ,* and M. A. VADAS

From the Clinical Research Unit of The Walter and Eliza Hall Institute of Medical Research and the 
Royal Melbourne Hospital, Victoria 3050, Australia

Delayed-type hypersensitivity (DTH) is a T cell-mediated reaction resulting in a mononuclear cell-rich inflammation and swelling that peak 24-48 h after local antigenic challenge. In spite of the extensive use of DTH to dissect regulatory (1), genetic (2), and molecular (3) aspects of in vivo T cell responses, little is known of factors that regulate the persistence of these reactions. It is thought that granulomata, such as those seen in tuberculosis and schistosomiasis, provide an example of prolonged DTH (4, 5) resulting from either the persistence of antigen (6) or from the absence of regulatory factors that promote the decay of DTH (7). An experimental model of prolonged T cell-dependent inflammation, in which a nonpersistent form of antigen is used, would allow an easier dissection of these regulatory factors and perhaps lead to maneuvers that abort some inflammatory responses. In this paper we show that administration of pertussigen, a purified protein from Bordetella pertussis (8), leads to intense and persistent DTH lesions in mice that are sensitized either actively or passively to protein antigens.

Materials and Methods

Mice. 8-10-wk old female mice of the BALB/cAn.Bradley WEHI strain were used throughout. All mice used were bred in specific pathogen-free conditions at The Walter and Eliza Hall Institute, and transferred to conventional mouse rooms before experiments.

Pertussigen. The crystalline preparation used in this work was made from supernatant fluids of 5-d old cultures of Bordetella pertussis by a modification of the method described by Cowell et al. (9). The preparation was extensively dialyzed against cold distilled water and then lyophilized. This dried material was kept in a desiccator at 4°C until needed. Solutions of dried pertussigen were made by weighing the desired amount and dissolving it in alanine formic acid buffer, pH 3.5 (6.78 g DL-a-alanine plus 0.7 g formic acid in 1 liter H2O), and immediately thereafter diluting it with an equal volume of 0.05 M Tris buffer, pH 8, containing 1 M sodium chloride. The solution, which usually contained 200 μg/ml, was stored at -15°C for a few months without losing its activity. For inoculation of mice, this stock solution was diluted in phosphate-buffered saline (PBS) to contain 2 μg/ml, and injected intravenously.

The BALB/c mice used in the present work responded to the intravenous administration of pertussigen with an increased susceptibility to a mixture of 200 μg histamine and 50 μg...
serotonin (SDs = 13 ng) and with a marked leukocytosis (over threefold the normal leukocyte count when 140 ng of pertussigen were given). These two actions are characteristic of pertussigen (10). In some experiments pertussigen was inactivated by placement in a boiling water bath for 20 min (10).

**Endotoxin.** The sample used was made from *B. pertussis* cells by the phenol-water method of Westphal et al. (11).

**Immunization.** Mice were immunized with an emulsion made of equal volumes of an antigen solution (1 mg/ml) and complete Freund's adjuvant (CFA) (Difco Laboratories, Michigan). A total volume of 0.1 ml per mouse was administered subcutaneously into the hind footpads and abdomen. The antigens used were keyhole limpet hemocyanin (KLH) (Calbiochem-Behring, La Jolla, CA) and 5X recrystallized egg albumin (EA) (ICN Pharmaceuticals, Inc., OH). In some experiments, 2 d before immunization, mice were injected with 100–150 mg/kg cyclophosphamide (CY) (Endoxan-Asta; Bristol Laboratories, Australia) subcutaneously into the nape of the neck.

**Challenge.** 6 d after immunization, the left pinnae of mice were subcutaneously injected with 10 μl of solution containing 1 mg/ml of antigen. The difference in ear thickness (increment) between the left and right pinnae was determined by measuring both ears with a spring-loaded micrometer (Mitutoyo Mfg. Co., Tokyo, Japan).

**Nonspecific Irritants.** Mice were injected in the left hind footpad with either 20 μl mineral turpentine (neat) or 50 μl of an emulsion of equal volumes of incomplete Freund's adjuvant (IFA) (Difco Laboratories) and isotonic sodium chloride. The increment in hind footpad thickness was determined in the same way as the ear increment.

**Cell Transfer.** To increase passive transfer of DTH (12) donor mice were treated with 150 mg/kg CY 2 d before immunization with KLH in CFA. 5 d later, mice were killed by cervical dislocation and the popliteal, inguinal, and para-aortic lymph nodes were excised. A single-cell suspension was prepared by teasing the lymph nodes through a fine stainless steel sieve. Eagle's minimum essential medium buffered with 20 mM Hepes (HEM) was used throughout. The cell suspensions were washed three times, the viable cells counted by eosin exclusion, and the appropriate number of cells was suspended in 0.2 ml HEM and injected intravenously into naive, syngeneic recipients, which were then challenged within 1 h.

**Treatment with Anti-Thy-1.2 and Complement.** Lymph node cells were prepared from CY and KLH-CFA-treated donors as above, washed three times and divided into two equal parts. One part was incubated for 30 min at 4°C with a rat monoclonal anti-Thy-1.2 (gift of Dr. L. Herzenberg) antibody purified from hybridoma culture supernatant. The other part was incubated in HEM. All the cells were then washed twice and incubated for 30 min at 37°C with rabbit serum absorbed extensively with mouse spleen cells as a source of complement, used at a final dilution of 1:20. The cells were then washed twice and counted. Each recipient in both the complement-alone and antibody plus complement groups received 10^7 cells.

**Histology.** In some experiments, sections of ears were stained with hematoxylin and eosin after fixation with 10% formalin.

**Results**

**Effect of Pertussigen on DTH in Immunized Mice**

Effect of Pertussigen on the DTH Response. Mice were treated with 400 ng pertussigen on the day of immunization with KLH in CFA and challenged 6 d later. 24 h after challenge, a significantly greater swelling was observed in the ears of pertussigen-treated mice compared with controls (Fig. 1). Over the next few days, the ear swelling in the pertussigen-treated mice continued to increase, reaching a maximum 4–7 d after challenge, whereas the control mice returned towards baseline levels. In six consecutive experiments similar findings were observed. At 24 h, there was a significant difference in every experiment (P < 0.02 to <0.001) between pertussigen-treated mice (mean increment in all experiments 282 μm) and sensitized groups not treated with pertussigen (160 μm). At 6 or 7 d, the difference was also significant in
Fig. 1. Time course of the effect of pertussigen on DTH responses. Groups of mice given pertussigen (●, ▲) or saline (○, ▼) were either immunized with KLH-CFA (●, ○) or left unimmunized (▲, ▼). 6 d later all groups were challenged with KLH and ear increments were measured on the stated days after challenge. Each point is the arithmetic mean of determinations in five mice. Vertical bars span 2 SEM.

### TABLE I

Effect of Pertussigen on Nonspecific Inflammatory Responses *

| Group | Substance injected | Pertussigen | 3 d† | 6 d | 9 d |
|-------|--------------------|-------------|------|-----|-----|
|       |                    |             | μm   |     |     |
| 1     | Turpentine         | +           | 1,320 ± 119$ | 1,100 ± 181 | 657 ± 158 |
| 2     | Turpentine         | −           | 1,148 ± 71  | 857 ± 63  | 615 ± 190 |
| 3     | IFA-saline         | +           | 864 ± 39  | 790 ± 57  | 696 ± 43  |
| 4     | IFA-saline         | −           | 882 ± 84  | 874 ± 114 | 828 ± 83  |

* Mice were injected in the left hind footpad with 20 μl of turpentine or with 50 μl of an emulsion of IFA and saline. Some groups were given pertussigen 400 ng i.v. on the same day.
† Peripheral blood white cell count per mm$^3$ at 3 d: group 1, 23.4 ± 1.9 ($\times 10^9$ mm$^{-3}$); group 2, 8.3 ± 1.1; group 3, 21.3 ± 1.2; group 4, 9.5 ± 0.9.
§ Arithmetic mean ± 1 SEM, 5 mice per group. The footpad thickness in untreated mice was 1,798 ± 28 μm. At all time points, there was no significant difference in the incremental footpad thickness between groups 1 and 2 or between groups 3 and 4 (calculated by Student's $t$ test, $P > 0.05$ not significant).

every experiment ($P < 0.01$ to $<0.001$) with a mean increment of 316 μm in pertussigen groups and 68 μm in sensitized controls. A significant swelling persisted for at least 6 wk after challenge. In control unimmunized mice treated with pertussigen and ear-challenged with KLH 6 d later, there was a small but significant ear swelling 6–12 d after challenge (Fig. 1). There was little reaction to KLH challenge in control mice given CFA, and the small delayed ear swelling seen in pertussigen-treated nonimmune mice did not occur.

To show that the effect of pertussigen was antigen specific, mice were injected with mineral turpentine or with an emulsion of IFA and saline. Since these substances were highly irritating, the footpad was chosen as the site of injection. Pertussigen injected intravenously on the same day had no significant influence on the footpad swellings at 3, 6, or 9 d after injection, in spite of the expected pertussigen-mediated leukocytosis (Table I). In contrast, when mice were injected in the footpad with antigenic substances such as KLH in CFA, the footpad increment was doubled by pertussigen at days 6 and 9 (J. J. Munoz and W. A. Sewell, manuscript in preparation).

**Specificity of the Pertussigen Effect.** Mice were immunized with either KLH
or EA in CFA and challenged with KLH, EA, or saline 6 d later. The results in Fig. 2 show some ear swelling in all groups at 4 h, with no effect of pertussigen. Thereafter mice challenged with the appropriate antigen developed ear swelling that was markedly increased by pertussigen. Challenge with the inappropriate antigen without

| Immunization | Challenge | Time After Challenge |
|--------------|-----------|----------------------|
| 1 KLH        | KLH       | 4h                  |
| 2 KLH        | EA        | 24h                 |
| 3 KLH        | NS        | 6d                  |
| 4 EA         | EA        | 4h                  |
| 5 EA         | KLH       | 24h                 |
| 6 EA         | NS        | 6d                  |

Fig. 2. Demonstration of the antigen specificity of the pertussigen effect on DTH. Mice were immunized with KLH or EA in CFA, and 6 d later challenged with KLH, EA, or normal saline (NS). Some groups received 400 ng of pertussigen on the day of immunization (open boxes) and other groups received saline (hatched boxes). The ear increments are shown 4 h, 24 h, and 6 d after challenge. Each value is the arithmetic mean in five mice, and bars represent one SEM above and below the mean.

Fig. 3. The effect of the dose of pertussigen on DTH. Varying doses of pertussigen were given to groups of mice on the day of immunization with KLH-CFA (○). Control mice (●) were given 400 ng of pertussigen on the same day. All groups were challenged 6 d later. Ear increments, measured 3, 6, and 8 days after challenge, had similar dose-response curves; those on day 3 are shown here. Each point is the arithmetic mean of measurements in five mice, and vertical bars span 2 SEM.

Fig. 4. The effect on DTH of the time of administration of pertussigen. Mice were immunized with KLH-CFA at day 0 and given 400 ng pertussigen at various time points (●). Immunized mice not given pertussigen are also shown (○). 6 d after immunization, mice were challenged with KLH, and the ear increments were measured 4 d after challenge. Each point represents the arithmetic mean of values in five mice, and the bars span 2 SEM.
pertussigen did not elicit a DTH response. KLH challenge in EA-immune mice was not enhanced by pertussigen, whereas EA challenge in KLH-immune mice was slightly increased by pertussigen. At the end of the experiment, the inappropriately challenged mice were challenged with the appropriate antigen in the opposite ear, and gave a strong DTH reaction.

**Effect of Dose of Pertussigen on DTH.** 400 ng of pertussigen at the time of immunization with KLH in CFA produced a substantial increase in the DTH response. Lower doses produced a weaker enhancement, with a demonstrable effect at doses as low as 25 ng (Fig. 3).

**Effect of Timing of Pertussigen on DTH.** To ascertain the optimal time of administration of pertussigen, mice were given 400 ng from 21 d before to 7 d after immunization. The mice were challenged 6 d after immunization and ear swellings measured 1-28 d later. The results at 4 d after challenge (Fig. 4) showed that pertussigen was most effective given on the day of immunization or 3 d later. Similar results were seen at 7 d after challenge, but from day 14 to day 28 mice given pertussigen 3 d after immunization had consistently higher ear swellings than mice given pertussigen on the day of immunization. For example, on day 28 the values were 200 ± 15 μm and 100 ± 19 μm, respectively (P < 0.01).

**Influence of Adjuvants and of CY on the Pertussigen Effect.** Mice were immunized with EA either emulsified with CFA (Table II, groups 1-5) or with the same quantity of antigen in saline (groups 6, 7), and were challenged with EA 6 d later. Some mice were also pretreated with CY (groups 4, 5). 400 ng of pertussigen or heat-inactivated pertussigen was given on the day of immunization (groups 2, 3, 5, 7). Pertussigen increased DTH to soluble EA or EA-CFA, with or without CY, 24 h and even more markedly 6 d after challenge. To investigate the role of lipopolysaccharide in these observations, the pertussigen preparation was heated for 20 min in a boiling

| Group | CY* | EA† | Pertussigen on day of immunization‡ | Incremental ear thickness after challenge |  |  |  |  |
|-------|-----|-----|-----------------------------------|------------------------------------------|---|---|---|---|
| 1     | −   | CFA | 0                                 | 154 ± 23†                               |  |  | 64 ± 8 |
| 2     | −   | CFA | 400                               | 280 ± 2                                 | <0.01 | 364 ± 39 | <0.001 |
| 3     | −   | CFA | 400 H**                           | 160 ± 2                                 | NS    | 88 ± 29  | NS    |
| 4     | +   | CFA | 0                                 | 212 ± 7                                 | 112 ± 11 |
| 5     | +   | CFA | 400                               | 286 ± 12                                | <0.001 | 390 ± 36 | <0.001 |
| 6     | −   | PBS | 0                                 | 116 ± 19                                | 90 ± 24  |
| 7     | −   | PBS | 400                               | 172 ± 13                                | <0.05  | 236 ± 9  | <0.001 |
| 8     | Challenge only (nonimmune) |     | 84 ± 23                           | 42 ± 5                                  |     |     |

* 100 mg/kg CY was given to some groups (+) 2 d before immunization.
† 50 μg of EA was given in either CFA or in PBS, subcutaneously.
‡ Pertussigen (400 ng) on the day of challenge enhanced DTH 6 d after challenge, but was ineffective at 24 h.
** Heat-inactivated pertussigen was heated in a boiling water bath for 20 min. Heated pertussigen given to mice treated with CY and EA-CFA was also ineffective.
†† Arithmetic mean ± 1 SEM. There were five mice per group.
□ P values are estimated by Student's t test. In groups 2 and 3, values are calculated by comparison with group 1; in group 5 with group 4; and in group 7 with group 6. NS, not significant, P > 0.05.
water bath, a treatment known to inactivate pertussigen but not lipopolysaccharide. The heat-treated material was ineffective in this system (Table II). In addition, 400 ng of \textit{B. pertussis} lipopolysaccharide had no effect on DTH (data not shown). Also notable was that CY, another agent that increases DTH, only had a marked influence at 24 h and, unlike pertussigen, CY was much less effective at 6 d. There was little or no additive effect when both CY and pertussigen were given.

\textbf{Histology.} Histologic examination demonstrated that there was no appreciable cellular infiltrate in ears taken 4 h after challenge, whereas a mononuclear infiltrate was present in immune mice at 24 h, with and without pertussigen. In the immune mice not treated with pertussigen, the cellular infiltrate waned by 6 d, whereas the infiltrate in the pertussigen-treated mice persisted and was still predominantly mononuclear (Fig. 5).

\textbf{Effect of Pertussigen on Passive Transfer of DTH.} To examine the effect of pertussigen on the recipients of sensitized cells, lymph node cells from CY-pretreated KLH-CFA-immunized mice were injected into nonimmune syngeneic recipients that were immediately ear challenged with KLH. These recipients developed an ear swelling that was maximal at 24 h and waned thereafter (Table III). It should be noted that ear swellings declined more slowly in the recipients of sensitized cells than in the immunized mice themselves. When pertussigen was given to recipients at the time of transfer, there was a small decrease in the 24 h reaction in some experiments. However, pertussigen significantly increased the swelling at later time points (Table III, experiment 1). The administration of pertussigen to donor mice increased the efficiency of cell transfer at 24 h but the reactions did not increase thereafter (experiment 2). In mice that did not receive cells, ear injection of KLH caused a small swelling that was not affected by pertussigen for the first 4 d, but increased slightly in the later

\textbf{Fig. 5.} Histologic appearance of ear sections stained with hematoxylin and eosin. \( \times 150 \). Mice were immunized with KLH-CFA and challenged with KLH 6 d later. The ears were taken 6 d after challenge. (Left) 400 ng pertussigen was given on the day of immunization. (Right) No pertussigen was given.
**TABLE III**

Influence of Pertussigen and Cyclophosphamide on the Passive Transfer of DTH to KLH *

| Experiment | Donors | Recipients | Cell number transferred | Ear increment (μm) after challenge |
|------------|--------|------------|-------------------------|-----------------------------------|
|            | CY     | P          |                         | Day 1   | Day 4   | Day 6   | Day 11  |
| 1          | +      | -          | +                       | 15 × 10⁶ | 190 ± 10** | 393 ± 25* | 553 ± 54* | 258 ± 36** |
|            | +      | -          | -                       | 15 × 10⁶ | 240 ± 7  | 225 ± 7  | 205 ± 20 | 118 ± 7   |
|            | -      | +          | Nil                     | 43 ± 10  | 60 ± 14  | 93 ± 11  | 90 ± 8   |
|            | -      | -          | Nil                     | 75 ± 7   | 40 ± 8   | 55 ± 15  | 40 ± 7   |
| 2          |        |            |                         | Day 1   | Day 2   | Day 4   |
|            | -      | +          | -                       | 10 × 10⁶ | 196 ± 12* | 215 ± 12* | 192 ± 17** |
|            | -      | -          | -                       | 10 × 10⁶ | 114 ± 4  | 96 ± 8   | 96 ± 14  |
|            | -      | -          | Nil                     | Nil     | 48 ± 2   | 33 ± 6   | 25 ± 3   |

* Donor mice were sensitized with KLH-CFA and treated with or without pertussigen (P) (400 ng on the day of immunization) or CY (150 mg/kg 2 d before immunization). Recipients in some groups were treated with pertussigen (400 ng on the day of transfer). 5 d after immunization, the stated number of lymph node cells was transferred. Ear thickness was measured on the stated days after challenge.
* Arithmetic mean ± 1 SEM, four to five mice per group. P values are determined by comparison of groups in which donors or recipients of sensitized cells received pertussigen with control groups which received sensitized cells. *, <0.001; **, <0.01.

**TABLE IV**

Effect of Anti-Thy-1.2 Antibody and Complement on the Transfer of DTH into Normal and Pertussigen-treated Recipients

| Treatment of donor cells* | Incremental ear thickness after challenge in recipients given: |
|---------------------------|---------------------------------------------------------------|
|                           | Pertussigen | Saline |
|                           | 24 h | 6 d | 24 h | 6 d |
| Anti-Thy-1.2 and complement | 37 ± 25 | 83 ± 9 | 47 ± 22 | 37 ± 21 |
| Complement alone | 177 ± 15 | 333 ± 9 | 155 ± 46 | 125 ± 16 |
| No cells | 45 ± 7 | 84 ± 25 | 32 ± 4 | 15 ± 4 |

* Lymph node cells from mice treated with CY and KLH-CFA were incubated with complement and antibody or complement alone as described in Materials and Methods. Recipients were treated with 400 ng pertussigen or saline 6 d before transfer.
* Arithmetic mean ± 1 SEM, with three to six mice per group.

measurements (experiment 1). The passive transfer into normal or pertussigen-treated recipients was characteristic of DTH in being abolished by monoclonal anti-Thy-1.2 and complement (Table IV).

**Discussion**

*B. pertussis* vaccine and impure extracts of the lymphocytosis-promoting factor of *B. pertussis* have previously been shown to have either facilitatory or inhibitory effects on
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DTH (10, 13). In this paper we show that pertussigen, a purified protein from *B. pertussis*, has several remarkable facilitatory effects on DTH in mice. First, it enhanced approximately twofold the magnitude of DTH measured 24 h after challenge (Fig. 1). Second, the peak intensity of DTH was changed from the normal 1–2 d to 4–7 d after challenge (Figs. 1, 2) at which time an approximately fivefold increase in intensity was evident. Third, DTH reactions with pertussigen persisted so that detectable ear swelling was present for at least 3 wk (Fig. 1) to as much as 6 wk (data not shown) after ear challenge, in comparison with control mice where it declined to near background levels by 6–7 d (Figs. 1, 2). It should be noted that the effect of pertussigen was unlike that of CY, another agent used for enhancing DTH. Although CY increased DTH at 24 h, these reactions declined thereafter (Table II), suggesting that these two agents have a dissimilar mechanism of action. In spite of this the pertussigen-promoted reactions had the characteristics of DTH, with a delayed onset (Fig. 2), antigen specificity (Fig. 2), H-2 restriction (data not shown), and typical histology (Fig. 5). Furthermore, inflammatory responses to irritant agents, not known to be antigenic, were not markedly enhanced by pertussigen, suggesting that its effect was primarily on antigen-driven inflammatory responses.

Besides the effect on immunized mice, pertussigen also caused a delayed increase in the intensity of DTH, when given to the recipients of sensitized cells. It was notable that no increase was evident in the recipients 24 h after ear challenge (Table III), suggesting that an immune reaction and the effects mediated by pertussigen had to coexist for a critical period for enhancement of DTH to be seen. T cells were at least in part responsible for this reaction as it was abolished by treatment of lymph node cells with anti-Thy-1.2 serum and complement (Table IV). In contrast to the treatment of recipients, pertussigen treatment of donor-sensitized cells did increase the magnitude of the 24-h ear reaction. However, this reaction did not increase on subsequent days (Table III). These findings suggest that the adjuvant-like effects of pertussigen on sensitized lymph node cells did not account for the delayed peak of intensity that occurred in pertussigen-treated recipients.

The pertussigen used in our experiments was purified to crystallization but it is possible that traces of contaminating substances could account for the biological activities. However, the most likely contaminant, endotoxin, had no significant effect on DTH (data not shown), making its role in this unlikely. Furthermore, the activity on DTH was destroyed by heating the preparation in a boiling water bath for 20 min (Table II), a treatment known to destroy other biological activities of pertussigen (10).

Pertussigen has several other biological effects, notably, induction of lymphocytosis with inhibition of lymphocyte homing to lymphoid organs (14), sensitization to histamine, enhancement of production of IgE, and increased incidence and severity of experimental autoimmune encephalomyelitis (15). The relationship of the mechanisms involved in pertussigen's effects on DTH to those of its other functions is currently under investigation, but the narrow time frame in which it exerts its maximal effect on DTH (Fig. 4) makes it unlikely that the phenomenon is totally explicable by its histamine-sensitizing property insofar as the latter has an extremely protracted time course (10). The lymphocytosis-promoting effect, which peaks 3 d after the administration of pertussigen, may well be associated with the effect on DTH. Furthermore, an increased number of mast cell precursors have been observed in pertussigen-treated mice (R. M. Crapper, W. A. Sewell, J. W. Schrader, and M. A.
Vadas, unpublished observations), suggesting that they may play a role in regulating the persistence as well as the development (16) of DTH.

At this stage, even without knowledge of the precise mode of action, we believe that pertussigen has great potential for enhancing the detection of sensitization by poor immunogens, e.g., soluble EA (Table II) and antidiagnostic sera (17). In addition it provides a model of prolonged T cell-dependent reactions that may lead to a better understanding of the regulation of chronic inflammation.

Summary

Pertussigen, a purified protein from *Bordetella pertussis*, was shown to increase delayed-type hypersensitivity (DTH) to protein antigens in mice. First, it caused an approximately twofold enhancement of the magnitude of 24-h DTH reactions. Second, the peak magnitude of DTH was delayed to 4–7 d after challenge, at which time it was five times more intense than in mice not receiving pertussigen. This reaction was antigen specific, and histologically was characterized by a dense mononuclear infiltrate. Third, pertussigen prolonged DTH so that it was still detectable 3–6 wk after challenge. The effect of pertussigen was seen only in antigen-driven reactions and was time and dose dependent, with 400 ng given 3 d after immunization resulting in the most prolonged reaction. The administration of pertussigen to the recipients of sensitized lymph node cells resulted in DTH that was more intense and prolonged than the reactions in control mice. Administration of pertussigen provides a model of prolonged and enhanced T cell-dependent inflammatory responses.

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