IMMUNE RESPONSE TO CHEMICALLY MODIFIED FLAGELLIN

II. EVIDENCE FOR A FUNDAMENTAL RELATIONSHIP BETWEEN HUMORAL AND CELL-MEDIATED IMMUNITY

BY C. R. PARISH, Ph.D.

(From the Department of Microbiology, The John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia)

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In the preceding paper (1) some chemical, antigenic, and biological properties of a series of acetoacetylated derivatives of Salmonella adelaide flagellin were described. Several chemical and antigenic tests revealed that as flagellin was acetoacetylated to increasing extents there was a steady decline in the affinity of the molecule for anti-flagellin antibodies. This loss in antigenic activity appeared to be accompanied on the one hand by a reduced capacity to initiate antibody formation but on the other hand by an enhanced ability to induce antibody tolerance. In this paper the antibody tolerance induced by these acetoacetyl derivatives is studied in more detail. Furthermore, the ability of these acetoacetyl derivatives to induce delayed-type hypersensitivity is reported. From these studies it was found that suppression of antibody formation in adult rats was accompanied by enhanced cell-mediated immunity. In fact, it appears that in adult animals antibody formation and cell-mediated immunity may well be opposing immunological processes. By contrast, tolerance induced in neonatal rats by acetoacetyl flagellin existed at the level of both humoral and cell-mediated immunity. Some preliminary aspects of this work have been reported elsewhere.1


Materials and Methods

Animals.—Randomly bred Wistar rats of either sex were used. Animals were either obtained directly from the Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, or bred in Canberra from Wistar breeders supplied by the Walter and Eliza Hall Institute. Both adult (6–10 wk of age) and neonatal rats were used.

Antigens.—Flagellin and polymerized flagellin from S. adelaide (strain SW 1338; H antigen—4g) and flagellin from S. typhimurium (strain SL 871; H antigen—1, 2) were prepared as described previously (2). A cyanogen bromide (CNBr) digest of S. adelaide flagellin was prepared and fractionated into fragment A and a mixture of fragments B, C, and D as described.
scribed elsewhere (3). The acetoacetylated derivatives of S. adelaide flagellin were prepared as described in the preceding paper (1). S. adelaide endotoxin (O antigen-35) was phenol extracted according to the method of Westphal et al. (4) Sheep red blood cells were obtained fresh and washed four times with saline before use. Hemocyanin (HCY)9 from Jasus islandii was kindly provided by our colleague Dr. R. E. Langman. HCY was acetoacetylated by a procedure similar to that described for flagellin (1). Briefly, HCY (5 mg/ml) was reacted at pH 8.5 with a 1000-fold molar excess of diketene for 6 hr at 20°C. After reaction the solution was dialyzed against distilled water and the number of acetoacetyl groups estimated spectrophotometrically. Using these reaction conditions 12.3 acetoacetyl groups/mole were attached to HCY.

The antigens were injected in saline either intraperitoneally (in neonatal tolerance experiments), intradermally into the flanks, or subcutaneously into the hind footpads. In some experiments antigen was emulsified in Freund’s complete adjuvant (FCA) and injected into the flanks or into the hind footpads. The routes of injection for each experiment are presented in the tables and accompanying text.

Antibody Estimations.—Antibody to flagellin was usually estimated by hemagglutination, using sheep erythrocytes sensitized with S. adelaide polymerized flagellin by a chromic chloride procedure.1 A few sera were also titrated using the technique of bacterial immobilization (2), but in all cases tested, the two techniques gave similar antibody titers.

Testing for Immediate, "Jones-Mote," and Delayed-Type Hypersensitivity.—Hypersensitivity reactions were determined by measuring the increase in footpad thickness following the injection of antigen in saline into the hind footpads. To elicit flagellin-specific reactions, usually 0.5 #g (50 #l) of flagellin was injected, although doses of flagellin as large as 100 #g elicited identical hypersensitivity responses. Two methods were used for estimating footpad swelling. (a) Eliciting antigen was injected into both hind footpads and footpad thickness was measured at zero time (prior to injection) and at various time intervals up to 48 hr post-challenge. Footpad swelling was estimated by subtracting the zero time reading from the later measurements.

(b) Antigen in saline was injected into the right hind footpad, saline alone into the left hind footpad. Footpad thickness was measured at various time intervals postchallenge and specific footpad swelling was determined by subtracting the thickness of the left hind footpad from the right.

In both cases footpad thickness was measured by a dial caliper gauge A02T (Schnelltaster, H. C. Kröplin GmbH, Schluchtern, Hessen, Germany). Both methods gave very similar hypersensitivity measurements, although the latter technique was eventually favored as it gave slightly more reliable measurements. For each experiment the method used for estimating hypersensitivity is indicated in the accompanying text. In several preliminary experiments reactions were elicited in the skin and specific increase in skin thickness was determined. Swellings comparable to the footpad technique were obtained but it was found that the footpad method was more convenient and accurate. It was assumed that in rats an immediate (Arthus) response peaked at 3 hr, a Jones-Mote response at 9 hr, and a delayed-type response at 24-48 hr (5).

Collection of Serum and Peritoneal Cells.—To obtain routine serum samples, rats were bled from the tail. To obtain larger quantities of serum for transfer experiments rats were exsanguinated and their serum pooled. Peritoneal cells were obtained by washing out the peritoneal cavities of rats with 5 ml of a solution of 5% foetal calf serum–phosphate-buffered saline (0.12 m NaCl-0.02 m sodium phosphate buffer, pH 7.0) containing 10 units/ml of heparin. The peritoneal cells were pooled and washed four times with the same medium but lacking

9 Abbreviations used in this paper: FCA, Freund’s complete adjuvant; HCY, hemocyanin; Krel, relative antigenic activity.
heparin. Finally, peritoneal cells (2 × 10^7 cells/rat in 0.5 ml) were injected intravenously into the lateral tail vein of recipient rats. Most rats yielded approximately 2.5-3.0 ml serum and 2.0-2.5 × 10^7 peritoneal cells.

Statistical Methods.—Standard errors of the means and P values were calculated using the Student's t test.

RESULTS

Humoral and Cell-Mediated Immune Responses to Flagellin.—Rats (seven per group) were injected with varying amounts of flagellin, either intraperitoneally in saline or subcutaneously in FCA (flank injection) and antibody titers subsequently determined. Four wk later hypersensitivity reactions were elicited by the injection of 0.5 μg of flagellin in saline into the right hind footpads. Footpad thicknesses were measured and footpad swelling was determined by subtracting the thicknesses of the left hind footpads which had received saline alone. Measurements were made at 3, 6, 9, 12, 24, and 48 hr postchallenge.

Fig. 1 depicts the time course of a hypersensitive response to flagellin in rats which had been sensitized with 1 μg of flagellin in saline. Flagellin sensitization...
induced no significant immediate or Jones-Mote hypersensitivity compared with control animals but did produce a small but highly significant delayed response \( (P = 0.01) \). It should be noted that control, nonsensitized animals, when injected with flagellin, exhibited substantial footpad swelling from 3 to 12 hr postchallenge (Jones-Mote response), but this reaction had completely subsided by 24 hr (Fig. 1). It was found that the Jones-Mote response was directed against trace amounts of salmonella endotoxin present in the flagellin preparations.

Table I summarizes the ability of various doses of flagellin in saline or FCA to induce immediate and delayed-type hypersensitivity. Only 100 \( \mu \text{g} \) of flagellin in FCA produced a strong immediate response, even though much lower doses of flagellin produced substantial antibody titers (e.g., 1 \( \mu \text{g} \) in FCA). There was a small but significant immediate reaction induced by 100 \( \mu \text{g} \) of flagellin in saline \( (0.02 > P > 0.01) \). In contrast, delayed-type hypersensitivity was induced to a similar extent by a wide range of flagellin doses. Furthermore the...
same level of delayed reactivity occurred when flagellin was injected in either saline or FCA.

In subsequent experiments it was demonstrated that the route of injection did not significantly influence the level of delayed-type hypersensitivity induced. Rats sensitized intraperitoneally or subcutaneously into either the flanks or hind footpads exhibited similar delayed reactivities. Also, identical delayed responses were observed when animals were sensitized and elicited in either the same or different sites.

**Humoral and Cell-Mediated Immune Responses to the Acetoacetylated Flagellins.**—The acetoacetylated derivatives of flagellin were tested for their ability to induce antibody formation, "antibody tolerance," and immediate and delayed-type hypersensitivity. Groups of adult rats (7-8/group) were injected into the hind footpads with 1 μg of flagellin or with 1 μg of one of the seven acetoacetylated flagellins in FCA. Control animals received a saline-FCA mixture. Thirty-five days later animals were challenged in the hind footpads with 1 μg of flagellin in saline (0.5 μg/footpad). Footpad thickness was measured at zero time (prior to challenge) and at 3, 8, 24, and 48 hr postchallenge, footpad swelling being estimated by subtracting the zero time thickness from the later measurements. Antibody titers against flagellin were measured at weekly intervals during the experiment. The seven acetoacetylated flagellin preparations were characterized by their content of acetoacetyl groups and their "relative antigenic activities" (Krel values—see preceding paper [1]) which were as follows: (a) 5.0 acetoacetyl groups/mole, Krel = 7.1 × 10⁻¹; (b) 8.0, 6.1 × 10⁻¹; (c) 10.8, 3.45 × 10⁻¹; (d) 14.6, 1.5 × 10⁻¹; (e) 16.8, 6.8 × 10⁻³; (f) 17.8, 1.5 × 10⁻⁶; (g) 20.6, 6.8 × 10⁻¹⁰.

**Humoral antibody responses to the acetoacetylated flagellins:** Figs. 2 and 3 plot the weekly antibody titers of rats which were primed with the different acetoacetylated flagellins and challenged 35 days later with unmodified flagellin. Fig. 4 relates antigenic activity to both primary and anamnestic antibody responsiveness, whereas Fig. 5 compares antigenicity with the extent of antibody tolerance induced. From the data presented in Figs. 2-5 the following observations were made and conclusions drawn:

(a) Acetoacetylation very readily destroyed the capacity of flagellin to induce a primary antibody response; in fact the 1.5 × 10⁻¹ to 6.8 × 10⁻¹⁰ preparations produced no detectable primary antibody (Figs. 2-4).

(b) As the extent of acetoacetylation increased, the ability of flagellin to induce immunological memory was also steadily reduced (Figs. 2-4). However, the heavily acetoacetylated preparations (6.8 × 10⁻³, 1.5 × 10⁻⁶, and 6.8 × 10⁻¹⁰ flagellins) did induce slight memory 7 days postchallenge when compared with unprimed control animals (Figs. 3 and 4).

(c) Very lightly substituted flagellin (5.0 acetoacetyl groups/mole, Krel =
7.1 $\times 10^{-1}$ induced primary and secondary antibody responses not significantly different from unmodified flagellin (Fig. 2). However, when injected in saline rather than FCA, this preparation did induce substantially lower antibody titers than flagellin.

![Graph](image)

**Fig. 2.** The ability of flagellin and its acetoacetylated derivatives to induce antibodies to flagellin (dose = 1 μg in FCA). Rats challenged on day 35 with 1 μg of unmodified flagellin in saline. Legend: flagellin (■—■); flagellin—5.0 acetoacetyl groups/mole, $K_{rel} = 7.1 \times 10^{-1}$ (△—△); flagellin—8.0, 6.1 $\times 10^{-1}$ (□—□); flagellin—10.8, 3.45 $\times 10^{-1}$ (▲—▲); flagellin—14.6, 1.5 $\times 10^{-1}$ (○—○). Control rats (●—●) primed with FCA and challenged on day 35 with flagellin (1 μg). Vertical bars represent standard errors of means.

(d) Adult rats which received one of the three more heavily acetoacetylated flagellins ($1.5 \times 10^{-1}$, $6.8 \times 10^{-3}$, and $1.5 \times 10^{-3}$ preparations) were rendered partially tolerant to a subsequent challenge of flagellin (Fig. 5). Significant antibody tolerance was induced by these preparations on the 14th, 21st, and 28th day after flagellin challenge ($P$ values < 0.001 for the 28 day time
point). Animals pretreated with the lightly substituted flagellins (7.1 $\times$ 10$^{-3}$, 6.1 $\times$ 10$^{-3}$, or 3.45 $\times$ 10$^{-3}$ preparations) exhibited prolonged immunological memory (Fig. 2).

(e) The most heavily acetoacetylated preparation of flagellin (20.6 aceto-
acetyl groups/mole, $K_{rel} = 6.8 \times 10^{-19}$, although producing slight immunological memory, induced no significant antibody tolerance, the 14-, 21-, and 28-day postchallenge titers being no different from control animals (Fig. 3).

**Hypersensitive responses to the acetocetylated flagellins:** The same rats which had been injected with the acetocetyl flagellins and which had their humoral antibody responses measured, were also tested for their ability to express immediate, Jones-Mote, and delayed-type hypersensitivity to flagellin (see experimental details earlier). To eliminate any nonspecific reactions (Fig. 1) the 3-, 24-, and 48-hr footpad swellings presented in Fig. 6 represent the net increases in footpad thickness over control unprimed rats which were elicited with flagellin, i.e., nonspecific (endotoxin?) swelling subtracted.

None of the acetocetyl flagellins induced significant immediate or Jones-Mote hypersensitivity (Fig. 6). This was not surprising, since no detectable antibody was produced by the more heavily acetocetylated preparations. In contrast, all treatments gave significant delayed-type hypersensitivity to

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**Fig. 4.** The relationship between the antigenic activity of the acetocetyl derivatives of flagellin and their ability to induce primary antibodies and immunological memory to flagellin. Legend: 35 day primary antibody titers to flagellin—priming = 1 $\mu$g of the various acetocetyl flagellins in FCA ($\square$); 7-day secondary antibody titers to flagellin—challenge = 1 $\mu$g of flagellin in saline ($\blacksquare$). The horizontal line (-----) represents the 7 day antibody response (after injection of 1 $\mu$g flagellin) of control rats which had been pre-injected with FCA. Vertical bars represent standard errors of means.
As the antigenic activity of flagellin was reduced by acetoacetylation, its capacity to induce the delayed response was steadily enhanced. In fact, except for the $7.1 \times 10^{-4}$ preparation, all of the acetoacetylated flagellins induced delayed reactions significantly higher than flagellin. This delayed swelling was still evident 48 hr postchallenge even though all responses had substantially fallen by this time point. Maximum delayed-type hypersensitivity was induced by the $1.5 \times 10^{-5}$ preparation (approximately four times higher than the flagellin response) (Fig. 6). Further loss in antigenic activity reduced the hypersensitivity response very significantly (i.e., $6.8 \times 10^{-10}$ preparation). This result is compatible with the finding that this preparation cannot induce antibody tolerance and suggests that very extensive acetoacetylation eventually destroys the immunological activity of flagellin.

Fig. 7 compares the antibody tolerance and delayed-type hypersensitivity induced by the various acetoacetyl flagellins. There clearly appears to be a

![Graph](image-url)
"mirror image" relationship between these two responses. When antibody tolerance exists, delayed-type hypersensitivity is enhanced whereas a strong antibody response is accompanied by weak cell-mediated immunity. Antigenic activity appears to be the factor which determines which one of these situations predominates.

**Humoral and Cell-Mediated Immune Responses to Other Forms of Flagellin.**

**Polymerized flagellin:** Flagellin (mol wt 40,000) readily polymerizes in the presence of high salt concentrations to form a filamentous structure similar to bacterial flagella (2). Antigenically, flagellin and polymerized flagellin ("polymer") are very similar (2). An experiment was performed to compare the ability of flagellin and polymerized flagellin to induce antibody formation and delayed-type hypersensitivity in rats.

Adult rats (7–8/group) were injected into the hind footpads with 1 μg of either flagellin or polymerized flagellin emulsified in FCA. Control animals received an FCA-saline mixture. Animals were challenged into the hind footpads 35 days later with 1 μg of flagellin in saline and footpad swelling was
measured as described in the preceding section. Antibody titers were measured at weekly intervals during the experiment.

It was found that, except for the 28 day time point, polymerized flagellin produced significantly higher primary antibody titers than flagellin (Fig. 8). In contrast, flagellin produced slight immunological memory to a flagellin challenge whereas polymer induced no memory. Table II presents the immediate and delayed-type hypersensitivity responses. No significant immediate response was produced by either flagellin or polymer. Polymerized flagellin also produced no delayed hypersensitivity whereas flagellin produced a significant delayed reaction ($P = 0.01$).

**Cyanogen bromide digest of flagellin:** Earlier work in this laboratory has demonstrated that all of the antigenic determinants of flagellin survive CNBr digestion and are, in fact, localized on the largest CNBr fragment (fragment A, mol.wt. 18,000) (6). Precise antigenic studies in the preceding paper (1) have demonstrated that CNBr-digested flagellin has a slightly reduced affinity for anti-flagellin antibodies ($K_{rel} = 5.7 \times 10^{-4}$). If antigenic activity is the
factor which determines immunogenicity, the CNBr digest should induce immune responses similar to an acetoacetylated flagellin of comparable antigenicity (i.e. flagellin substituted with 8.0 acetoacetyl groups/mole, $K_{ac} = 6.1 \times 10^{-4}$). The experimental protocol was identical to that described earlier for the acetoacetylated flagellins and polymerized flagellin; in fact the immunogenicity of the CNBr digest was determined at the same time as these other antigens.

Both the CNBr digest of flagellin and $6.1 \times 10^{-4}$ acetoacetyl flagellin produced comparable primary and secondary antibody titers (Fig. 9). However, both preparations induced very significantly lower primary antibody responses ($P < 0.001$) than unmodified flagellin. After flagellin challenge all treatments gave similar antibody levels, although there was a suggestion that rats primed
with either the CNBr digest or $6.1 \times 10^{-4}$ acetoacetyl flagellin produced slightly lower titers. No significant immediate-type hypersensitivity was induced by any of the treatments (Table III). In contrast, all preparations produced highly significant delayed reactions ($P < 0.001$ at 24 hr time point). Both CNBr-digested and acetoacetylated flagellin produced identical delayed hypersensitivity but hypersensitivity which was significantly higher than that induced by unmodified flagellin ($0.02 > P > 0.01$ at 24 hr time point).

Specificity of the Cell-Mediated Immunity Induced by Flagellin and Acetoacetylated Flagellin.—Adult rats (7-8/group) were primed in the flanks with 1 $\mu$g of either flagellin or acetoacetylated flagellin ($16.8$ acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$) emulsified in FCA. Four wk later hypersensitivity reactions were elicited by the injection of the different antigen preparations into the right hind footpads. Footpad thicknesses were measured at 3, 24, and 48 hr postchallenge and footpad swelling determined by subtracting the thicknesses of the left hind footpads which had received saline alone. No Arthus responses were elicited by any of the antigens and when delayed hypersensitivity was observed it peaked at 24 hr. The different eliciting antigens and 24-hr footpad swellings are listed in Table IV.

The delayed-type hypersensitivity induced by acetoacetylated flagellin was flagellin specific. Unrelated antigens such as sheep red blood cells and HCY did not elicit the reaction. Similarly, the delayed response was not directed against the acetoacetyl group as acetoacetylated HCY failed to elicit a response. In addition, a dose of $S. adelaide$ endotoxin ($0.002 \mu$g) corresponding to the possible endotoxin content of an eliciting dose ($0.5 \mu$g) of flagellin elicited no delayed hypersensitivity.
In contrast, the delayed-type hypersensitivity induced by either flagellin or acetoacetylated flagellin was elicited by a wide range of flagellin preparations. For example, a 0.5 μg dose of the most heavily acetoacetylated preparation of flagellin (20.6 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-40}$) could readily elicit both flagellin and acetoacetyl flagellin–induced hypersensitivity. However, unmodified flagellin was the most efficient at eliciting the delayed response. As little as 0.01 μg of flagellin produced a significant delayed reaction ($P < 0.001$) whereas comparable doses of acetoacetylated flagellin failed to elicit a response.

Flagellin from *S. typhimurium* (SL871) which antigenically only weakly
cross-reacts with *S. adelaide* (SW1338) flagellin could also elicit the delayed-type hypersensitivity induced by acetoacetylated flagellin. Furthermore, fragment A (mol. wt. 18,000), the CNBr fragment of *S. adelaide* flagellin which carried all of the serological specificities of the protein (6), could also elicit this delayed response. In contrast, the nonantigenic CNBr fragments (fragments B, C, and D) failed to elicit any hypersensitivity.

**Effect of Adjuvants on the Delayed-Type Hypersensitivity Induced by Acetoacetylated Flagellin.**—Numerous workers have demonstrated that many protein antigens must be injected emulsified in FCA before they will induce detectable delayed-type hypersensitivity (7). The ability of acetoacetylated flagellin (16.8 acetoacetyl groups/mole, *K*_rel = 6.8 × 10⁻⁸) to induce delayed reactivity in saline or FCA was determined. Adult rats were sensitized in the flanks with 1 μg of antigen either in saline or FCA and elicited 4 wk later in the right hind footpads with 0.5 μg flagellin. Delayed-type hypersensitivity was measured as in the preceding section.

Animals primed with acetoacetyl flagellin in saline produced good delayed-type hypersensitivity (Table V). However, higher delayed reactions tended to be induced when the antigen was injected in FCA. Results from two sepa-
Rats were primed in the flanks with 1 µg of either *S. adelaide* (SW1338) flagellin or acetoacetylated flagellin (16.8 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$) emulsified in FCA. Four wk later hypersensitivity reactions were elicited in the hind footpads with the different antigen preparations.

* Standard error of mean.

† The relative antigenic activity ($K_{rel}$) of the preparation compared with unmodified flagellin (1).

§ Purified fragments from a CNBr digest of *S. adelaide* flagellin (3).

‖ Flagellin from *S. typhimurium* (SL871).

¶ Measurement: cells, not µg.

<table>
<thead>
<tr>
<th>Priming antigen (1 µg)</th>
<th>Eliciting antigen</th>
<th>Dose of eliciting antigen</th>
<th>24 hr footpad swelling (3rd hr mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCA alone</td>
<td>Flagellin</td>
<td>0.5</td>
<td>0.4 ± 0.3*</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Flagellin</td>
<td>0.5</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.5</td>
<td>2.9 ± 0.4</td>
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<tr>
<td>Flagellin</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.5</td>
<td>3.0 ± 0.5</td>
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<tr>
<td>Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.5</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>&quot; &quot; Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.01</td>
<td>3.3 ± 0.5</td>
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<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.5</td>
<td>5.6 ± 0.6</td>
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<tr>
<td>&quot; &quot; Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.01</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>&quot; &quot; Flagellin (16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.5</td>
<td>5.8 ± 0.8</td>
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<td>&quot; &quot; Fragment A§</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.01</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>&quot; &quot; Fragment B, C, and D§</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.25</td>
<td>5.2 ± 0.2</td>
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<tr>
<td>&quot; &quot; Flagellin (SL871)‖</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.25</td>
<td>0.8 ± 0.2</td>
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<tr>
<td>&quot; &quot; Sheep red blood cells</td>
<td>Flagellin (20.6 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$)</td>
<td>0.5</td>
<td>5.9 ± 0.4</td>
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<td>&quot; &quot; HCY</td>
<td>Sheep red blood cells</td>
<td>1 × 10^4¶</td>
<td>0.2 ± 0.3</td>
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<td>&quot; &quot; HCY (12.3 acetoacetyl groups)</td>
<td>Sheep red blood cells</td>
<td>5</td>
<td>0.7 ± 0.3</td>
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<tr>
<td>&quot; &quot; HCY (12.3 acetoacetyl groups)</td>
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<td>5</td>
<td>0.7 ± 0.3</td>
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<td>&quot; &quot; S. adelaide endotoxin</td>
<td>Sheep red blood cells</td>
<td>0.002</td>
<td>0.2 ± 0.1</td>
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</table>
rate FCA experiments are presented in Table V. In one experiment a 24 hr swelling of 7.0 was observed, which was significantly higher than the saline-primed animals (P = 0.01), whereas in another experiment the FCA treatment did not produce a significant enhancement (24 hr swelling 5.7; 0.1 > P > 0.05). There was no indication that trace amounts of S. adelaide endotoxin had an adjuvant effect.

**Ability of Cells and Serum to Transfer Delayed-Type Hypersensitivity Induced by Acetoacetylated Flagellin.**—Peritoneal cells and serum were obtained from normal rats or from rats which had been sensitized 4 wk previously with

### TABLE V

**Delayed-Type Hypersensitivity Reactions Induced in Rats by Acetoacetylated Flagellin Injected in Saline or in FCA**

<table>
<thead>
<tr>
<th>Priming antigen (1 µg)</th>
<th>Injecting solution</th>
<th>Footpad swelling (1/16th mm) at times after eliciting dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>Acetoacetyl flagellin (16.8 acetoacetyl groups/mole, K&lt;br&gt;&lt;sub&gt;rel&lt;/sub&gt; = 6.8 × 10&lt;sup&gt;-3&lt;/sup&gt;)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Saline</td>
<td>4.2 ± 0.4&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>FCA&lt;sup&gt;§&lt;/sup&gt;</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>FCA&lt;sup&gt;§&lt;/sup&gt;</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>Saline + 0.004 µg S. adelaide endotoxin</td>
<td>4.2 ± 0.6</td>
</tr>
</tbody>
</table>

Rats were primed in the flanks and elicited in the hind footpads with flagellin (0.5 µg) four weeks later.

<sup>*</sup>The relative antigenic activity (K<sub>rel</sub>) of the preparation compared with unmodified flagellin (1).

<sup>‡</sup>Standard error of mean.

<sup>§</sup>Two separate experiments.

1 µg of acetoacetyl flagellin (16.8 acetoacetyl groups/mole, K<sub>rel</sub> = 6.8 × 10<sup>-3</sup>) in FCA. Normal rats were then injected with either peritoneal cells (2 × 10<sup>7</sup> cells/rat, intravenously) or serum (2.5 ml/rat, intraperitoneally) and 30 min later hypersensitivity reactions elicited by the injection of 0.5 µg of flagellin in saline into the right hind footpads. Footpad thicknesses were measured at 3, 24, and 48 hr and footpad swelling was determined by subtracting the thicknesses of the left hind footpads which had received saline alone. It was observed that delayed-type hypersensitivity was very efficiently transferred from sensitized rats by peritoneal cells (approximately 60% transfer of the delayed response of donor rats). In contrast, peritoneal cells and serum from normal rats or serum from sensitized rats failed to transfer delayed responsiveness (see Table VI).
Inability of Specific Antibody to Suppress the Expression of Delayed-Type Hypersensitivity.—Data presented in this paper have indicated an inverse relationship between serum antibody levels and delayed-type hypersensitivity to flagellin. One possible explanation of this phenomenon is that serum antibody is actively suppressing the expression of delayed-type hypersensitivity. If this were the case, passive administration of antibody should suppress delayed reactivity, whereas peritoneal cells from animals with high antibody titers should readily transfer delayed responsiveness.

Peritoneal cells and serum were obtained from normal rats and from rats which had been sensitized 4 wk previously with 1 µg of polymerized flagellin (polymer) in FCA. The serum samples were injected into recipient rats which had been sensitized 4 wk earlier with 1 µg of acetoacetyl flagellin in FCA (16.8 acetoacetyl groups/mole, \( K_{rel} = 6.8 \times 10^{-3} \)), whereas the peritoneal cells were transferred into normal rats. Delayed-type hypersensitivity was elicited and measured as described in the preceding section.

In agreement with data presented earlier (see Table II and Fig. 8), polymerized flagellin produced high antibody titers against flagellin but failed to induce detectable delayed-type hypersensitivity (see Table VII). Peritoneal cells from polymer-primed animals also failed to transfer delayed responsiveness to normal rats. Furthermore, serum from polymer-immunized rats in no way suppressed the expression of delayed-type hypersensitivity induced by acetoacetyl flagellin (Table VII).

### Table VI

<table>
<thead>
<tr>
<th>Transfer agent</th>
<th>Amount</th>
<th>Footpad swelling (3/4th mm) at times after eliciting dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>Normal peritoneal cells</td>
<td>2 ( \times ) 10(^7) cells</td>
<td>1.0 ± 0.3*</td>
</tr>
<tr>
<td>Cellular immune peritoneal cells†</td>
<td>2 ( \times ) 10(^7) cells</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Normal serum</td>
<td>2.5 ml</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Cellular immune serum‡</td>
<td>2.5 ml</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

* Standard error of mean.
† Peritoneal cells and serum from rats which had been sensitized 4 wk previously with 1 µg of acetoacetyl flagellin (16.8 acetoacetyl groups/mole, \( K_{rel} = 6.8 \times 10^{-3} \)) in FCA. The hypersensitivity responses in recipient rats were elicited with 0.5 µg of flagellin.
‡ The delayed-type hypersensitivity response in actively sensitized rats. Similarly sensitized rats were used as cell and serum donors.

Ability of Acetoacetylated Flagellin to Induce Immunological Tolerance in Neonatal Rats.—Acetoacetylated flagellin (16.8 acetoacetyl groups/mole,
K_{rel} = 6.8 \times 10^{-3}) was injected into groups of rats (6–8/group) in amounts of 1 \mu g, three times weekly, beginning within 24 hr of birth. After 8 wk of injections these rats were challenged into the flanks with either 1 \mu g of flagellin in FCA or 1 \mu g of acetoacetyl flagellin in FCA (16.8 acetoacetyl groups/mole, K_{rel} = 6.8 \times 10^{-3}). Control groups of 8-wk-old rats which had not received a course of injections of acetoacetyl flagellin were also challenged in the same way. Antibody titers were measured prior to challenge and at weekly intervals for 4 wk postchallenge. The prechallenge and 28-day postchallenge titers are presented in Table VIII. Four wk after the FCA challenges all animals were elicited with 0.5 \mu g of polymerized flagellin in FCA. The hypersensitivity responses were elicited with 0.5 \mu g of flagellin.

<table>
<thead>
<tr>
<th>Priming antigen</th>
<th>Priming dose</th>
<th>Transferred material</th>
<th>Footpad swelling (5 mm) at times after eliciting dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>Polymerized flagellin in FCA</td>
<td>1</td>
<td>Nil</td>
<td>0.7 ± 0.3*</td>
</tr>
<tr>
<td>Acetoacetyl flagellin in FCA (16.8 acetoacetyl groups/mole, K_{rel} = 6.8 \times 10^{-3})</td>
<td>1</td>
<td>Nil</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-polymer\textsuperscript{†} antiserum (2.5 ml) (HA titer = 1/1000)</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-polymer\textsuperscript{†} peritoneal cells (2 \times 10^7)</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal peritoneal cells (2 \times 10^7)</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Standard error of mean.

\textsuperscript{†} Peritoneal cells and serum from rats which had been immunized 4 wk previously with 1 \mu g of polymerized flagellin in FCA. The hypersensitivity responses were elicited with 0.5 \mu g of flagellin.

After 8 wk of injection with acetoacetyl flagellin no rats had detectable antibody (see Table VIII). In agreement with data presented earlier (Figs. 3–5), rats which were challenged with 1 \mu g of acetoacetyl flagellin in FCA...
40 RELATIONSHIP BETWEEN HUMORAL AND CELLULAR IMMUNITY

(16.8 acetoacetyl groups/mole) also produced no detectable antibody up to 28 days postchallenge. Pretreatment of animals from birth with acetoacetyl flagellin induced highly significant antibody tolerance to a subsequent challenge of flagellin (approximately 90% suppression of antibody formation, \( P < 0.001 \)). Furthermore, neonatal injections of acetoacetyl flagellin also rendered rats completely tolerant at the level of cell-mediated immunity. These animals were unable to produce detectable delayed-type hypersensitivity against flagellin or acetoacetyl flagellin (\( P < 0.001 \) in both cases). Thus, acetoacetyl flagellin induces tolerance in neonatal rats at the level of both humoral and cell-mediated immunity, whereas the same preparation in adult rats produces only antibody tolerance.

**TABLE VIII**

*Induction of Tolerance in Neonatal Rats by Heavily Acetoacetylated Flagellin*

<table>
<thead>
<tr>
<th>Initial course of injections*</th>
<th>Antibody titer before second injection</th>
<th>Antibody titer (28 day)</th>
<th>Delayed-type hypersensitivity (28 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μg acetoacetyl flagellin†</td>
<td>&lt;2.5 Flagellin</td>
<td>96</td>
<td>0.3 ± 0.1§</td>
</tr>
<tr>
<td>Nil</td>
<td>&lt;2.5 Flagellin</td>
<td>960</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>1 μg acetoacetyl flagellin</td>
<td>&lt;2.5 Acetoacetyl flagellin†</td>
<td>&lt;2.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Nil</td>
<td>&lt;2.5 Acetoacetyl flagellin†</td>
<td>&lt;2.5</td>
<td>6.8 ± 0.6</td>
</tr>
</tbody>
</table>

* Rats injected three times weekly for 8 wk prior to challenge.
† 16.8 acetoacetyl groups/mole, \( K_{rel} = 6.8 \times 10^{-3} \).
§ Represents 24 hr footpad swelling (1/10th mm) following elicitation with 0.5 μg of flagellin in saline. Standard errors of means are included.

**DISCUSSION**

Many workers have demonstrated that the immune response to an antigen can be both humoral and cellular in nature. However, the exact relationship between these two immune responses is not well understood, although several laboratories have demonstrated that selective suppression of either delayed-type hypersensitivity or antibody formation can be achieved. For example, Asherson (8, 9) and others (10–16) reported that animals pretreated with antigen in saline failed to develop delayed-type hypersensitivity to a subsequent challenge of the same antigen in adjuvant. In contrast there was a variable and usually slight effect on the antibody response. This phenomenon has been referred to variously as "immune deviation" (8), "split tolerance" (14), and "preimmunization tolerance" (13) and has also been likened to the Sulzberger-Chase phenomenon (15). On the other hand, it has been observed that antibody tolerance to tuberculin (17) and lysozyme (E. Benjamini, personal
In this paper a series of acetoacetyl derivatives of flagellin were studied which had slightly-to-greatly reduced affinities for anti-flagellin antibodies. It was found that loss in the antigenic activity of flagellin was accompanied by a reduced capacity of the molecule to initiate antibody formation but an enhanced ability of the protein to induce flagellin-specific cell-mediated immunity and antibody tolerance. From these results it can be concluded that in this system there seems to be an inverse relationship between cell-mediated immunity and humoral antibody responsiveness. In fact, it appears that antigenic activity (presumably the affinity of antigen for the receptors on cells) is the factor which determines which response predominates. Thus, antigen with high affinity for cell-bound receptors induces antibody formation, whereas low affinity antigen induces cell-mediated immunity and antibody tolerance.

In support of this concept was the finding that a CNBr digest of flagellin induced identical humoral and cellular immune responses to an acetoacetylated flagellin of comparable antigenic activity. It was also of particular interest that a similar dose of polymerized flagellin, a particulate form of the flagellin molecule, was a very efficient stimulator of antibody production but induced no detectable delayed-type hypersensitivity.

Other laboratories have also demonstrated that the chemical modification of antigens can greatly alter their immunogenicity. Benacerraf and Gell (18) reported that a range of protein antigens substituted with either picryl, acetyl, or ethoxymethylene-phenyloxazolone groups tended to induce protein specific delayed-type hypersensitivity more effectively than the native protein. In contrast, no antibodies could be detected against the protein carrier, although antibody was produced against the haptencic groupings. Furthermore, it has been recently demonstrated that carboxymethylated lysozyme, although producing no detectable antibodies against lysozyme, can induce both delayed-type hypersensitivity and antibody tolerance to lysozyme (E. Benjamin, personal communication). On the other hand, methylated serum albumins induced delayed hypersensitivity which was essentially conjugate specific (19).

Numerous workers have proposed that the antigenic determinants for humoral and cell-mediated immunity differ (7, 20, 21). This proposal has been based on the observation that the delayed-type hypersensitivity induced by a hapten-carrier complex is conjugate specific, whereas the antibodies produced are hapten specific. However, the data presented in this paper suggests that both humoral and cell-mediated immunity can be directed against the same antigenic determinant but that the specificity requirements for delayed hypersensitivity are much less than those required for antibody formation. This phenomenon is most clearly demonstrated in Fig. 7, where loss in antigenic
activity and induction of antibody tolerance is paralleled by an enhanced cellular immune response. This concept is further supported by the fact that heat-denatured proteins are as effective as native proteins in provoking delayed hypersensitivity in spite of the fact that denaturation greatly modifies their antigenicity (22). Assuming the much lower specificity requirements for delayed hypersensitivity, one would predict that many antigens which are closely related structurally, although showing little or no cross-reacting antibodies, should produce cellular immunity which strongly cross-reacts. In fact, S. adelaide and S. typhimurium flagellin, although producing very weakly cross-reacting antibodies, cross-react strongly at the cellular immunity level (Table IV). Gell and Benacerraf (22) reported a similar cross-reactivity between serum albumins. Furthermore, a similar phenomenon should exist for antibody tolerance as it appears that the specificity requirements for antibody tolerance are also much less than those required for antibody formation. It has already been demonstrated that the induction of neonatal tolerance with S. adelaide flagellin produces partial antibody tolerance to a range of other, antigenically different, flagellins (23). Similarly it has been reported that there is no straightforward correlation between the specificity of antigen-antibody interaction and that of cross-tolerance between a range of synthetic polypeptide antigens (24). In fact, it could be argued that antigenic competition is actually a reflection of very weak cross-reaction between structurally related antigens, i.e., partial antibody tolerance.

There is ample evidence that the 24-hr footpad swellings induced by the acetoacetyl flagellins represent genuine delayed hypersensitivity. The 24 hr response was transferred by cells and not by serum (25) (Table VI) and the histology of the reaction was characteristic of a delayed response. Furthermore, there was no evidence that delayed reactivity was induced by trace amounts of unmodified flagellin present in the acetoacetylated preparations (26) as low doses of flagellin failed to give delayed hypersensitivity (Table I). The possibility of antibody suppressing the expression of delayed hypersensitivity was also eliminated (Table VII).

The use of flagellin as an immunogen has several unique features and advantages which are as follows: (a) Flagellin is an excellent inducer of humoral antibodies, submicrogram doses in saline producing good antibody titers (27). (b) Flagellin induces comparable delayed hypersensitivity when injected in either saline or FCA, although the delayed response observed is comparatively small (Table I). Furthermore, FCA only slightly enhances the delayed hypersensitivity induced by the acetoacetyl flagellins (Table V). These findings are in direct contrast to studies with many other antigens where it has been demonstrated that FCA is essential for the induction of delayed-type hypersensitivity. (7) Trace amounts of S. adelaide endotoxin present in flagellin preparations may play an adjuvant role, although attempts to demonstrate
the adjuvant effect of endotoxin have failed (see Table V). (c) The acetoacetyl flagellins can efficiently induce antibody tolerance when injected in either saline (1) or FCA (Fig. 5). (d) Because flagellin is very rapidly eliminated from the circulation (28) and so little antigen is required to sensitize animals, there is no risk of desensitization occurring in immune animals. The fact that as little as 0.01 μg of flagellin can elicit a delayed response (Table IV) indicates that desensitization may readily mask delayed responsiveness in other systems where large quantities of slowly eliminated antigen are needed to immunize.

One of the most striking features of this study was the differentiation between neonatal and adult tolerance. Neonatal tolerance induced by acetoacetylated flagellin existed at the level of both humoral and cell-mediated immunity, a phenomenon which has been observed for a range of other antigens (29–31). In contrast, acetoacetyl flagellin in adult rats only produced tolerance at the level of antibody formation and the cellular immune response was greatly enhanced. In a subsequent publication it will be demonstrated that “cellular immunity tolerance” exists in animals which are expressing a strong antibody response. These results strongly suggest that antibody tolerance in a mature (adult) immune system exists at the “B” (berus-equivalent) cell level, whereas tolerance in neonatal animals probably exists at the level of both the “T” (thymus-dependent) and B cells. Presumably thymus-dependent cells undergo a period during their differentiation when tolerance to “self antigens” can be more readily induced.

In conclusion, a hypothesis will be briefly outlined which attempts to explain the phenomena reported in this paper. The concept is schematically presented in Fig. 10 and considers a mature (adult) immune system. To construct the hypothesis three basic assumptions are made:

(a) Both the B and T cell populations bear receptors on their surface which reflect the specificity of the immune response which they will ultimately express.

(b) Cell-cell interaction between the B and T cells is essential for the induction of antibody formation. There is ample evidence that this interaction occurs (32–36) but the exact nature of the cooperation is uncertain. There may be transfer of nonspecific factor(s) or translocation of specific genetic information (Fig. 10). The latter situation makes it mandatory that the B cells passively acquire their receptors.

(c) A “threshold energy of binding” of antigen with cell-bound receptors must be achieved before a cell is activated. Thus, the “activation” of cells would be an all or none phenomenon.

In Fig. 10 it is postulated that when antigen binds to either T or B cells and achieves the appropriate threshold energy of binding, the T cells are activated

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RELATIONSHIP BETWEEN HUMORAL AND CELLULAR IMMUNITY

into proliferation and the production of cell-mediated immunity whereas the B cells are rendered "tolerant." In contrast, interaction between the two cell lines with antigen acting as a molecular bridge, results in antibody formation and cellular immunity tolerance. Thus, the affinity of antigen for the receptors on cells is of crucial importance in determining the degree of cell-cell interaction and therefore the magnitude of the antibody response. If one lowers the affinity of an antigen for cellular receptors by chemical modification or CNBr digestion, one rapidly destroys the ability of the antigen to induce antibody formation. In contrast, all that is needed to induce antibody tolerance and cell-mediated immunity is that the modified antigen attains the threshold energy of binding required to activate cells. This hypothesis readily explains the observation that the specificity requirements for antibody tolerance and delayed hypersensitivity are much lower than those required for antibody formation. It is also consistent with the observation that low doses of antigen preferentially induce cell-mediated immunity (26) and antibody tolerance (37), whereas particulate antigens (e.g., polymerized flagellin) preferentially develop humoral antibodies.

SUMMARY

Flagellin (mol.wt. 40,000) from S. adelaide organisms and a series of aceto-acetyl derivatives of flagellin were tested for their ability to induce humoral
and cell-mediated immunity in adult rats. It was found that unmodified flagellin was an excellent inducer of antibody formation but a poor inducer of delayed-type hypersensitivity. In contrast, increasing acetoacetylation steadily destroyed the ability of flagellin to initiate antibody formation but enhanced the capacity of the molecule to induce flagellin-specific cell-mediated immunity and antibody tolerance. In fact, it appeared that in adult rats antibody formation and cell-mediated immunity may well be opposing immunological processes. Furthermore, the affinity of the acetoacetyl flagellins for anti-flagellin antibodies appeared to determine the type of immune response which predominated. High affinity antigen produced antibody formation whereas low affinity antigen induced cell-mediated immunity and antibody tolerance. The importance of affinity was further evidenced by the fact that a CNBr digest of flagellin induced humoral and cellular immune responses identical to an acetoacetylated flagellin of comparable antigenic activity. From these studies it was proposed that both humoral and cell-mediated immunity can be directed against the same antigenic determinants but that the specificity requirements for delayed hypersensitivity (and antibody tolerance) are less than those required for antibody formation.

Some remarkable immunological features of the flagellin system were revealed. Flagellin induced comparable delayed-type hypersensitivity when injected in either saline or FCA. Furthermore, FCA only slightly enhanced the delayed responses induced by the acetoacetyl flagellins and in fact these preparations produced antibody tolerance whether injected in saline or adjuvant.

Finally, in contrast to the adult tolerance induced by the acetoacetylated flagellins, which existed only at the antibody level, tolerance in neonatal rats existed at the level of both humoral and cell-mediated immunity. This finding is the first indication of a fundamental difference between neonatal and adult tolerance.

The significance of these findings is discussed in the light of current immunological concepts and a hypothesis proposed to explain these phenomena.

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REFERENCES


