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Immune Adherence by the Fourth Component of Complement

Abstract. *An immune complex becomes reactive in immune adherence after the specific binding of the fourth component of complement (C4). Immune adherence produced by the fourth component of complement is indistinguishable from classical immune adherence in terms of all parameters tested, except that it is entirely independent of the participation of the second and third components of complement.*

Immune adherence (IA) is the term used by Nelson (*1*) to describe the complement-dependent adherence of immune complexes to certain nonsensitized indicator particles, such as human erythrocytes. It has been widely used as a sensitive in vitro test for the detection of antigen, antibody, or bound complement components (*2*); it has also received some attention as a method for demonstrating isoantibody or preformed immune complexes in tissue sections (*3*), on isolated lymphocytes (*4; 5*, p. 223), or on tumor cells (*6*). Nishioka and Linscott first showed that the generation of an IA reactive complex required the specific binding of only the first four components—C1, C2, C3, and C4—of the complement system (*7*). Among these components, C1 and C2 are required only to mediate the specific binding to the immune complex of C4 and C3, respectively. While IA reactivity was readily evident after the fixation of small amounts of C3, it could not be demonstrated before the binding of this component. Furthermore, several investigators have shown that there is direct dependence of IA on the number of bound C3 molecules per cell (*5*, p. 245; *8*). There is thus little doubt that bound C3 is capable of mediating the IA reaction.

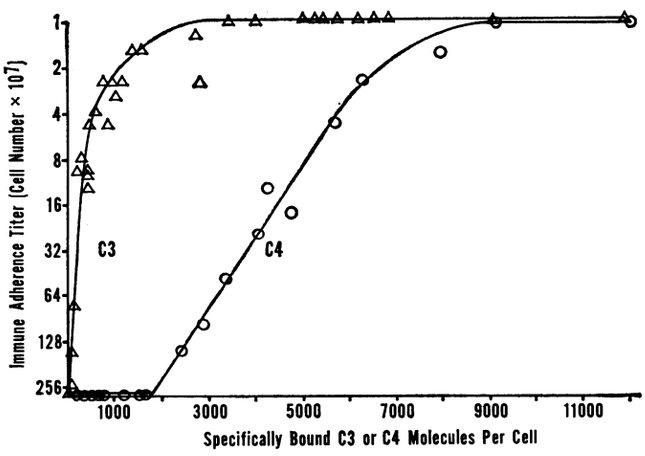
Recent studies indicate that specifically bound C4 can produce IA which is indistinguishable from classical IA, although it is entirely independent of C3. The reactivity of C4 in IA first became evident when it was observed that concentrated preparations of highly purified C4 were consistently reactive in IA.

For the study of C4-dependent IA (*9*), sensitized sheep erythrocytes (EA) bearing C1 (EAC1) were added to dilutions of purified C4 (*10*) to allow formation of EAC1,4. For the generation of C3-dependent IA, the intermediate complex EAC1,4 was added to dilutions of a mixture of purified C2 (*11*) and C3 (*12*) where C2 was present in excess. Human type O erythrocytes were subsequently added as indicator particles to dilutions of washed, water-hemolyzed EAC1,4 and EAC1,-4,2,3 (*13*). The agglutination titer was appraised by inspection of the sedimented erythrocytes. Coombs-type agglutination tests with antiserums directed against C3 or C4 and various complement intermediates were also performed (*14*).

Approximately 30 preparations of highly purified C4 were tested for their ability, in conjunction with EAC1, to produce IA. These preparations were IA reactive; furthermore, the resulting IA titer was directly dependent upon the C4 concentration in the reaction mixture. No reactivity was evident upon omission of C4, nor was IA produced with C4 preparations which had become inactivated with respect to hemolytic activity of C4. The reaction was also abolished upon heat denaturation (60°C, 30 minutes) or hydrazine (0.015*M*, 37°C, 45 minutes) treatment. A requirement for active C1 for the generation of IA reactivity was indicated by the observations that completely negative reactions resulted on omission of C1, after C1 removal from EAC1 by treatment with 0.02*M* EDTA, or after abrogation of C1 reactivity on EAC1 by treatment with $10^{-2}M$ diisopropylfluorophosphate. These studies indicate that IA reactivity results only after interaction of active C1 with active C4.

The data presented in Fig. 1 show the IA reactivity of ^{125}I-labeled, specifically bound C4 on EAC1,4 (*15*). This reactivity was first evident at 2500 to 3000 bound C4 molecules per cell and maximum reactivity was reached with about 9000 C4 molecules per cell. Between these values the IA titer was a function of the number of C4 molecules per cell. For comparison, Fig. 1 also shows the relation between the number of molecules of specifically bound C3 on EAC1,4,2,3 and the IA titer. For the preparation of EAC1,-4,2,3 only IA negative EAC1,4 complexes were used; that is, cells which contained less than 2500 C4 molecules per cell. Very few molecules of specifically bound C3, approximately 60 per cell, were required to produce a positive IA reaction. Between 60 and about 3000 C3 molecules per cell, where maximum reactivity was reached, the IA titer was dependent on the number of bound C3 molecules per cell. Both IA-positive EACl,4 and EACl,-4,2,3 were undiminished in reactivity after treatment with either EDTA or diisopropylfluorophosphate.

The extreme sensitivity of IA in detecting cell-bound C3 indicated that contamination of purified C4 with traces of C2 and C3 and resulting generation of a few C1,4,2,3 sites must be ruled out as the explanation for the IA reactivity of EAC1,4. One reaction of comparable sensitivity to IA in the detection of cell-bound C3 is the agglutination of cells bearing C3 by potent antiserums to this component. Therefore, numerous preparations of IA-positive EAC1,4 were examined for reactivity with antiserum to C3. Agglutination was not observed. The results of some of these experiments are shown in Fig. 2 which demonstrates the maximum IA reactivity obtained with four different EAC1,4 preparations as well as the completely negative patterns observed when the same cells were tested with antiserum to C3. By contrast, in parallel experiments,



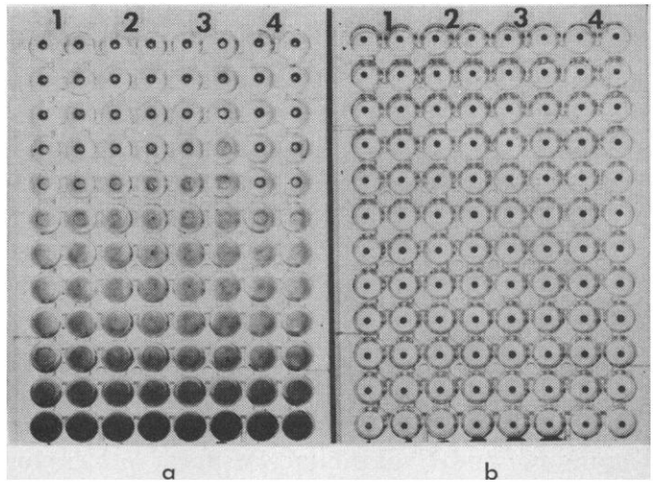


Fig. 1 (left). Number of molecules of specifically bound C3 (—△—) and C4 (—○—) necessary to produce immune adherence. The tests were performed with ^{125}I-labeled C3 and C4 bound to EAC1,4,2 and EAC1, respectively. Fig. 2 (right). (a) Immune adherence reactivity of four EAC1,4 preparations. The preparations were diluted in twofold steps from an initial concentration of 1×10^9 cell/ml. The endpoint was approximately 1×10^7 cell/ml, indicating maximum reactivity. (b) Coombs-type agglutination tests with monospecific antiserum to C3 performed with the same EAC1,4 preparations. The antiserum was diluted in twofold steps beginning at a 1:50 dilution. The reaction is entirely negative.

EAC1,4,2,3 were agglutinated by high dilutions of the same antiserum to C3. Another approach was also used to confirm the apparent lack of a C2 and C3 requirement for the production of C4-dependent IA. In these studies, a C4 preparation was either incubated with *p*-chloromercuribenzoate ($5 \times 10^{-4}M$) or heated (56°C, 30 minutes) in order to inactivate C2 as a possible contaminant. These treated C4 preparations retained full IA reactivity when tested with EAC1 and human erythrocytes. In a similar fashion, C4 was treated with reagents which inactivate C3, such as trypsin (1 percent, by weight; 20°C; 1 minute) and the complex consisting of cobra factor and C3 inactivator. Neither procedure diminished the IA reactivity of the C4 preparation. By contrast, in simultaneous experiments, a mixture of C2 and C3, after being heated or treated with *p*-chloromercuribenzoate, with trypsin, or with the C3-inactivator complex, lost its ability to confer IA reactivity upon IA-negative EAC1,4. Finally, the addition of an excess of either purified C2 or C3 to the C4 preparation did not enhance the IA reactivity.

In additional experiments, IA due to C4 was abolished after treatment of the human erythrocytes with trypsin or formaldehyde (*2*) as well as by replacement of the human erythrocytes by rabbit, sheep, or guinea pig erythrocytes. Immune adherence due to C3 or C4 could not be differentiated by the pattern of the sedimented erythrocytes, although the pattern produced by C4 often tended to collapse upon prolonged incubation. Microscopic examination of either C4- or C3-dependent IA revealed co-agglutination of the sheep and human erythrocytes.

The results described show that C4 does have the ability to produce immune adherence without the participation of either C2 or C3. In terms of all factors tested, including a requirement for an immune complex bearing specifically bound complement components and a trypsin- and formaldehyde-sensitive receptor present only on certain indicator particles, C3-produced IA and C4-produced IA are indistinguishable. It is thus probable that the same human erythrocyte receptor and similar forces are involved in immune adherence attributable to either component.

The nature of the interaction between bound complement and human erythrocytes in immune adherence is unknown. Previous studies indicated that it is not electrostatic (*16*). Certain observers have postulated an enzyme-dependent bond (*17*). The results here rule out this possibility unless the enzyme is present on the indicator cells: C4 does not possess known enzyme activity. It is more likely that this bond is formed after the uncovering of reactive groups on C3 and C4 by the action of the C1 and C4,2 enzymes, respectively. The third and fourth components are very similar proteins with respect to physicochemical properties, a common susceptibility to inactivation by the same chemical reagents, and the release of small fragments by the action of their respective activating enzymes. Thus it is not improbable that a similar or common reactive group is exposed on both proteins during complement action, and this group is capable of interacting with the IA receptor on the human erythrocytes.

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9. Immune adherence tests were performed in microtiter plates (Cooke Engineering). Twenty-five microliters of the complement intermediate complexes (4×10^7 cell/ml) were added to an equivalent volume of dilutions of the test component. After this mixture was intermittently shaken for 15 minutes at 37°C, 25 μl of human erythrocytes (8×10^7 cell/ml) in ethylenediaminetetraacetate (EDTA) buffer was added, and the plates were incubated with shaking for 15 minutes at 37°C. The cells were permitted to settle for 30 to 45 minutes at 37°C.
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13. These tests were performed in microtiter plates with 25 μl of dilutions of water-hemolyzed EAC1,4 or EAC1,4,2,3 prepared with the isolated components and 25 μl of human erythrocytes (8×10^7 cell/ml). This system reaches maximum reactivity at a concentration of 1×10^7 EAC1,4 or EAC1,4,2,3 per milliliter.
14. Coombs-type agglutination tests were performed in microtiter plates. Twenty-five microliters of complement bearing complexes (1×10^8 cell/ml) were added to dilutions of monospecific antiserums to C3 or C4. After being shaken intermittently for 15 minutes at 37°C, the cells were permitted to settle at 37°C for 30 to 45 minutes. The titer was appraised by inspection of the pattern of the sedimented erythrocytes.
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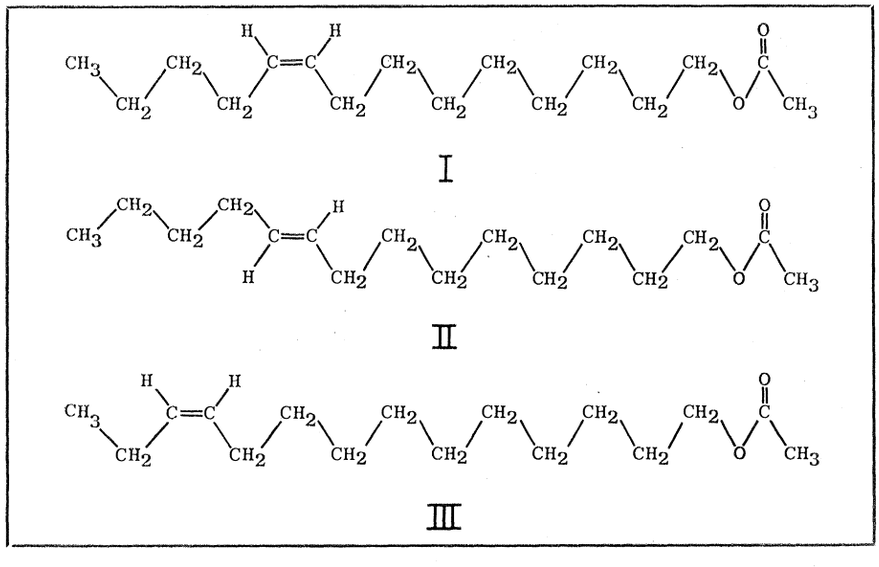
Sex Pheromone Specificity:

Taxonomic and Evolutionary Aspects in Lepidoptera

Abstract. *Sex pheromone specificity is the only obvious reproduction isolating mechanism for two tortricid species and two gelechiid species. Pheromones of the gelechiid species are* cis-*9-tetradecenyl acetate and* trans-*9-tetradecenyl acetate. Male response of one gelechiid species is inhibited by the pheromone of the other species. This could be important for sympatric evolutionary saltation.*

Sex pheromone specificity is one of several ways by which insect species can achieve reproductive isolation under natural conditions. In cases where pheromones are apparently nonspecific, other factors, such as variations in mating rhythms, seasonal cycles, host plant selection, and geographical distribution, can serve to isolate the species (*1*). If several insect populations appear similar in these biological considerations and in morphological detail, the determination of sex pheromone specificity would provide excellent support for the reproductive isolation of these populations in a nondimensional system. Reproductive isolation under natural conditions in turn "supplies a completely nonarbitrary criterion for the determination of species status of a population" (*2*). We have used sex pheromone specificity as a taxonomic principle in two examples of morphologically similar species of Lepidoptera by determining specificity with crude pheromone extracts in one case, and with synthetic pheromone compounds in the other. The pheromone compounds in the latter case were previously unknown for both species in the pair, and therefore, represent two new pheromone identifications.

The first example involves two tortricids, *Archips mortuanus* and *Archips argyrospilus*. Although Freeman (*3*) considered them sibling species, Powell (*4*) later suggested that the former is merely a darker form of the latter. In addition to possessing indistinguishable morphological adult characters, the two forms have the same seasonal cycles, feed on the same hosts (apple trees), and have indistinguishable egg masses and young larvae. An intensive comparative study (*5*), however, revealed differences in the ultimate larval instar that enables the two forms to be separated in field collections. Histological study (*5*) of the adult female genitalia showed that the gland producing pheromone is very similar in both forms; the glands are situated dorsally in the intersegmental membrane between the eighth and ninth abdominal segments as a convoluted, eversible sac of highly secretory, cuboidal-to-columnar, prismatic epithelium.



Studies of sex pheromone specificity (*6*) were conducted with crude pheromone extracts obtained by pulverizing the two terminal abdominal segments from 180 female *A. argyrospilus* moths in one batch and 80 female *A. mortuanus* in another. The moths were reared from ultimate instar larvae collected in stands of wild apple trees. A sample containing ten female equivalents of pheromone was placed on a 4-cm dental wick located within each trap (355.2 ml drinking cups lined with sticky polybutene). Sets of traps, each set consisting of an *A. argyrospilus* and an *A. mortuanus* trap located within 1 m of each other, were hung in the apple trees during moth flight. The traps with *A. argyrospilus* extract attracted 203 male *A. argyrospilus* and no *A. mortuanus* adults on the same nights that traps with *A. mortuanus* extract attracted 82 male *A. mortuanus* and 10 *A. argyrospilus* adults. The occurrence of these ten strays may be due to the high level of *A. argyrospilus* infestation encountered, and to the proximity of the *A. argyrospilus* traps and wild females. Clock-traps (*7*) charged with crude pheromone extracts were used to determine the circadian rhythm of male activity for each form. The two forms were attracted at essentially the same hours, so the only obvious isolating factor would be sex pheromone specificity. Our evidence for this specificity supports the conclusion that these are separate species.

The second example involves two species of Gelechiidae. The two species are morphologically similar and exhibit identical four-dot wing patterns, although one form, *Bryotopha similis* (B1), appears grayish and the other form (B2) more yellowish (*8*). The