

Immunologic responses of experimental animals to green algae

I. Leonard Bernstein, M.D., Guillermo V. Villacorte, M.D., and Robert S. Safferman, Ph.D., Cincinnati, Ohio*

Three common strains of green algae have proven to be highly immunogenic in experimental animals. Antisera obtained from these animals were used to study cross-reactivity among 6 strains of green algae and several nonalgal antigens. Results of gel diffusion and direct PCA studies revealed a limited pattern of cross-reactivity. However with the use of PCA and hemagglutination-inhibition techniques, broader patterns of cross-reactions were obtained. In addition, the possibility of common antigenic determinants between green algae and certain fungi has been raised.

Previous investigations demonstrated that a significant number of skin reactions occurred in atopic patients tested with green algal extracts.¹⁻³ This skin reactivity may be equated with reaginic-type antibodies since the majority of the individuals tested showed skin-sensitizing antibodies which could be passively transferred to normal recipients.³ Direct provocation of several of these atopic individuals with dilute solutions of aerosolized algal antigen resulted in significant bronchial mucosal responses which included instances of severe wheezing.³ The possible role of green algae as etiological agents in human respiratory allergies and the relative paucity of serologic data on these organisms have led us to examine their immunologic effects in experimental animals. The sensitization experiments in animals were designed to elucidate possible immunologic relationships among the green algae as well as between these algae and other allergens.

From the Division of Immunology, Allergy Section, Department of Internal Medicine, University of Cincinnati Medical Center and the Federal Water Pollution Control Administration, U.S. Department of Interior.

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MATERIALS AND METHODS

The green algae strains used in this study were axenic cultures of *Chlorella vulgaris* IU 263,* *Chlorella pyrenoidosa* SEC 121, *Chlorococcum botryoides* SEC 91, *Chlorococcum macrostigmatum* IU 109, *Scenedesmus basilensis* SEC 83, and *Ankistrodesmus falcatus* var. *acicularis* SEC 28. Methods for their maintenance, culture, and extraction have been previously described.³ Nonalgal antigens were also extracted in Coca's solution (pH 8.0), with the exception of the bacterial filtrate (Immunovac) which was prepared in cresol-saline solution at pH 7.2.

Antisera to *C. vulgaris*, *C. pyrenoidosa*, and *S. basilensis* extracts were prepared by injecting rabbits with two 0.5 ml. aliquots of a homogenized suspension containing one part of algal extract (1:40 dilution) and 2 parts of Freund's complete adjuvant. Each animal received 2 intramuscular, 2 subcutaneous, and 2 additional intramuscular injections for a period of 3 weeks. All rabbits were bled from the first through the eighth week after the last injection.

Hemagglutination was attempted initially by adsorption of algal extracts upon sheep erythrocytes treated with tannic acid. However, hemolysis occurred during the sensitization phase. Formalinized erythrocytes⁴ were then used and it was determined that 1:250 dilutions of *C. vulgaris* and *S. basilensis* and a 1:100 dilution of *C. pyrenoidosa* resulted in optimum passive sensitization of sheep red cells. A modified method of Daniel and Stavitsky⁴ was used for hemagglutination-inhibition tests. In this procedure 0.1 ml. of 1:250 or 1:100 dilution of all antigens was incubated with 0.1 ml. of antisera for 10 minutes at 37° C. prior to addition of sensitized sheep red cells. Hemagglutination was read after 16 hours and the reciprocals of these titers were subsequently used in the interpretation of data. Antisera with hemagglutinating activity were treated with 0.1M 2-mercaptoethanol according to the method of Porter.⁵

Precipitating antibodies were determined by double-gel diffusion.⁶ Titrations of algal antigens at 1:40, 1:400, and 1:4,000 dilutions against respective rabbit antisera revealed that the 1:40 antigen dilution gave the most dense and reproducible precipitin bands. Therefore these concentrations were used in all subsequent experiments. Cross-reactions were studied by placing rabbit antisera in the center well and various antigens in the peripheral wells. These antigens included the 6 algal extracts as well as *Alternaria*, *Monilia*, *Aspergillus fumigatus*, a mixed bacterial filtrate, and extracts of ragweed, grass, and tree pollens.

Fluorescent antibody studies were carried out by the method of Campbell and associates.⁷ The purified gamma globulin fractions obtained from rabbits immunized to *C. vulgaris*, *C. pyrenoidosa*, and *S. basilensis* were conjugated with 0.5 mg. fluorescein-isothiocyanate-Celite (FIC) per milligram protein. These conjugated antisera were incubated in tubes at 37° C. for 30 minutes with a 2 per cent suspension containing both whole and fragmented cells of sonicated algal preparations.³ A similar study was also carried out with the nonalgal antigens previously described. Specimens were washed 5 times in cold borate-saline buffer to exclude soluble constituents from the final preparations. After air dry-

*Culture numbers refer to the algal culture collections at Indiana University (IU), Bloomington, Ind., and Federal Water Pollution Control Administration (SEC), Cincinnati, Ohio.

ing, samples were examined with a Leitz fluorescent microscope with the use of a 2.5 mm. Euphos eyepiece filter and a 2 mm. UG-1 external filter.

Passive cutaneous anaphylaxis (PCA) was studied in albino guinea pigs which were challenged intravenously with various antigens and Evans blue dye 6 hours after intracutaneous injections of rabbit antisera.⁸ PCA inhibitory experiments were carried out with 0.05 ml. portions of 1:10 dilution of algal antisera which were incubated for 30 minutes with 0.05 ml. of the appropriate antigens diluted 1:40. This mixture was injected intracutaneously, and the animals were challenged intravenously in the manner previously described.

Hemolytic effects of algal extracts in vitro were examined with red blood cells of rabbit, sheep, and human origin. The anticoagulant effects of *C. vulgaris* extracts upon human blood were studied by the Lee-White⁹ and partial thromboplastin time¹⁰ methods. Short-term in vivo studies were performed in 6 rabbits. Blood counts, hematocrits, serum bilirubins, and Lee-White clotting times were determined 2, 5, and 24 hours after injections of 5 ml. of 1:40 *C. vulgaris* extract. Erythrocyte counts and hematocrit readings were also carried out in guinea pigs before and 2 hours after intravenous injections of 1 ml. of 1:40 *C. vulgaris* extracts.

RESULTS

Gel-diffusion studies

Rabbit precipitins appeared 4 weeks after initial immunization. As seen in Fig. 1, 2 to 3 bands were formed between the various antisera and their homologous antigens. These bands were observed within 72 hours. Bands resulting from cross-reactions appeared later and in some cases became well defined only 2 to 3 weeks after the plates were prepared. Nevertheless the final intensity was approximately equal to the intensity of homologous bands. *C. botryoides* cross-reactions appeared within one week while *C. pyrenoidosa* and *C. macrostigmatum* cross-reactions became well defined at the end of 2 weeks.

When *C. vulgaris* antiserum was placed in the center well, a band of non-identity was obtained against *C. botryoides* antigen. *S. basilensis* antiserum

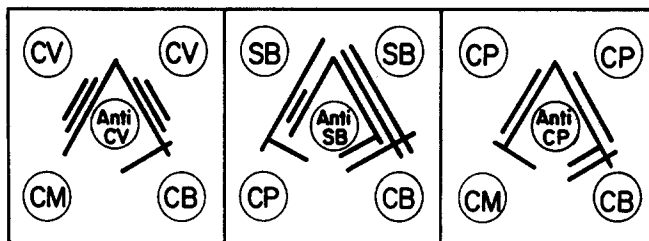


Fig. 1

Diagrams of double-gel-diffusion studies with *C. vulgaris* (anti CV), *S. basilensis* (anti SB), and *C. pyrenoidosa* (anti CP) antisera in center wells and *C. vulgaris* (CV), *S. basilensis* (SB), *C. pyrenoidosa* (CP), *C. botryoides* (CB), and *C. macrostigmatum* (CM) antigens in peripheral wells. Each homologous antigen-antibody system was analyzed with 2 different antigen preparations.

formed bands of partial identity with *C. pyrenoidosa* and *C. botryoides*. A band of nonidentity to *C. botryoides* was also observed. A band of partial identity developed between *C. pyrenoidosa* antisera and extracts of *C. botryoides* and *C. macrostigmatum*. A band of nonidentity also appeared between *C. pyrenoidosa* antisera and *C. botryoides*. None of the other algal antigens exhibited cross-reactivity to the 3 antisera, nor were cross-reactions observed between these antisera and mixed bacterial filtrate, fungi, or pollens of ragweed, grass, and trees.

Since *C. botryoides* formed bands against all 3 algal antisera, another gel-diffusion preparation was studied in which *C. botryoides* antigen was placed in the center well and 3 algal antisera in peripheral wells. In this case a concentric band of identity appeared close to the central well. The relative position of this band appeared to be similar to the location of various bands of difference which *C. botryoides* formed against all antisera in the individual gel experiments shown in Fig. 1. An additional band of identity also formed between the antigen *C. botryoides* and the antisera of *S. basilensis* and *C. pyrenoidosa*. This band appeared to be similar to the bands of partial identity shown in Fig. 1.

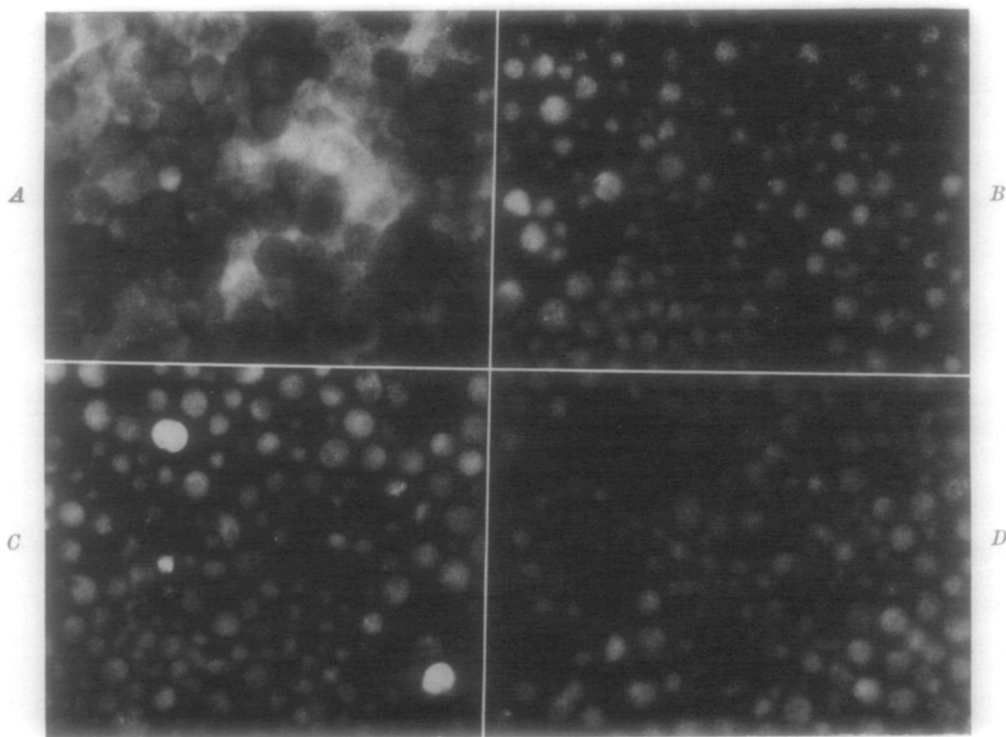


Fig. 2

Fluorescent antibody studies with sonicated *C. vulgaris* preparations containing both whole and fragmented cells. *A*, Algae + FIC conjugated gamma globulin antibody to *C. vulgaris*. *B*, Algae + specifically adsorbed FIC conjugated gamma globulin antibody. *C*, Algae + FIC conjugated normal rabbit gamma globulin. *D*, Algae + borate buffer. The nonspecific blue fluorescence which appeared in control preparations *B*, *C*, and *D* was easily differentiated from the light green fluorescence of preparation *A*.

Specificity of gel-precipitin reactions was studied by absorption experiments in which the 3 antisera were absorbed with homologous and heterologous antigens. Gel plates were set up in which the central wells contained the algal antigens and the peripheral wells the unabsorbed antisera and supernatant fluids of antisera absorbed with the algal and fungal antigens after the precipitates were removed. In these experiments algal-extract preparations inhibited formation of homologous precipitin bands while absorption with heterologous antigens and homologous whole-cell preparations did not demonstrate such an effect. However absorption with heterologous antigens did remove cross-reacting bands.

Fluorescent antibody study

Whole cells and subcellular components of *S. basilensis*, *C. botryoides*, and *C. macrostigmatum* exhibited a high degree of autofluorescence. However, background blue-green fluorescence of *C. vulgaris*, *C. pyrenoidosa*, and *A. falcatus* was minimal, thereby permitting fluorescent antibody studies with these organisms. Specific fluorescent reactions were demonstrated when *C. vulgaris* (Fig. 2) as well as *C. pyrenoidosa* were incubated with their respective labeled antisera. It should be noted that the cellular materials in Fig. 2, A tend to coalesce and that the bulk of fluorescence appears to be associated with the cellular fragments. This type of localization was expected since the technique of extract preparation apparently excluded the bulk of cell-wall antigen. In contrast, both clumping and specific fluorescence are not visible in the control preparations (Fig. 2, B, C, D). If whole cells alone are used as the substrate rather than sonicated cell preparations, the intensity of fluorescence diminishes significantly. Such structural variability in intensity of immunofluorescence has also been described for *Aspergillus flavus*.¹¹ No evidence of cross-reactivity was observed when the 3 fluorescent antisera were incubated with disrupted cell preparations of either *C. vulgaris*, *C. pyrenoidosa*, *A. falcatus*, or with any of the nonalgal antigens studied.

Passive cutaneous anaphylaxis

For direct PCA, algal extracts of *C. vulgaris*, *S. basilensis*, and *C. pyrenoidosa* diluted 1:40 were utilized for intravenous challenges. Optimum reactions were obtained when experiments were carried out with 1:10 dilutions of the 3 rabbit antisera. As shown in Table I, a definite cross-reaction was observed between *C. pyrenoidosa* antigen and *S. basilensis* antiserum. On the other hand, *S. basilensis* antigen failed to cross-react with *C. pyrenoidosa* antiserum.

In inhibition experiments with rabbit antisera, the highest dilutions at which complete blocking with homologous antigens occurred were 1:4,000 for *C. pyrenoidosa* and 1:400 for both *C. vulgaris* and *S. basilensis*. With respect to cross-reactivity, this technique demonstrated a wider spectrum than the direct PCA approach. As illustrated in Table II, in contrast to direct PCA, all 6 algal antigens tested showed some evidence of cross-inhibition. Moreover inhibitory effects were also noted with the fungal antigens, *Alternaria* and *Aspergillus*. Cross-reactions in Table II exhibit irregularity in that some inhibitory antigens show all or none effects while others show only partial effects. This irregular response

Table I. Cross-reactions in PCA guinea pig experiments

Rabbit antisera	Challenging antigen		
	C. vulgaris	C. pyrenoidosa	S. basileusis
<i>C. vulgaris</i>	7/7*	0/8	1/7
<i>C. pyrenoidosa</i>	0/7	8/8	0/7
<i>S. basileusis</i>	0/7	7/8	7/7

*Numerator denotes positive PCA reaction of 6 mm. or greater and the denominator indicates the total number of animals tested.

Table II. Inhibition in PCA guinea pig experiments

Inhibiting antigen	Rabbit antisera inhibited in vitro								
	C. vulgaris*			C. pyrenoidosa			S. basileusis		
	Com- plete	Partial	None	Com- plete	Partial	None	Com- plete	Partial	None
<i>C. vulgaris</i>	16	0	0	3	1	4	3	0	3
<i>C. pyrenoidosa</i>	5	0	2	12	0	0	3	0	3
<i>S. basileusis</i>	5	1	1	6	1	1	10	0	0
<i>C. botryoides</i>	0	0	6	2	1	3	2	0	3
<i>C. macrostigmatum</i>	4	3	2	6	0	3	4	0	3
<i>A. falcatus</i>	0	0	4	0	2	2	1	1	1
<i>Aspergillus</i>	1	0	3	1	1	3	0	1	2
<i>Alternaria</i>	2	2	3	0	2	4	0	1	5
<i>Monilia</i>	0	0	4	0	0	4	0	0	3

*Challenging homologous antigen (0.5 ml. of 1:40 dilution) was given intravenously 6 hours after intracutaneous application of antigen-antibody mixtures. Numbers refer to the individual guinea pigs tested and reactions were graded as follows: complete inhibition = absence of cutaneous bluing; partial inhibition = cutaneous bluing varying from 0 to 80 per cent of the diameter obtained in direct PCA control; none = cutaneous bluing of 80 per cent or more of the diameter of the direct PCA control.

can be attributed in part to concentration variability often encountered in different preparations of crude extracts. In addition it was not possible to ascertain the optimal inhibitory concentration for all antigens since the maximum concentrations of test antigens were employed throughout.

Hemagglutination

C. vulgaris and *S. basileusis* antisera showed hemagglutinating titers of 1:5,120, whereas a titer of 1:640 was obtained with *C. pyrenoidosa* antisera. Among these antisera and their respective antigens, direct cross-reactivity was observed only in the case of *S. basileusis* antiserum which reacted against both *C. vulgaris* and *C. pyrenoidosa* antigens. The inhibition studies presented in Table III appear to confirm this relationship, since hemagglutinating activity of the *S. basileusis* system was completely blocked by *C. vulgaris* and *C. pyrenoidosa* antigens. The inhibition technique also indicated the presence of cross-reactions to other algae. In addition, one of the fungi (*Alternaria*) partially inhibited hemagglutinating antibody specific for *C. pyrenoidosa* and *S. basileusis*. Treatment of the 3 antisera with 0.1M 2-mercaptoethanol resulted in complete loss of hemagglutinating activity, suggesting IgM-type antibodies.¹² Some findings in

Table III. Hemagglutination inhibition experiments

Reciprocal of maximum hemagglutinating titer			
Inhibiting antigen	Antiserum—homologous antigen system		
	C. vulgaris	C. pyrenoidosa	S. basilensis
<i>C. vulgaris</i>	0	320	0
<i>C. pyrenoidosa</i>	320	0	0
<i>S. basilensis</i>	320	80	0
<i>C. botryoides</i>	2,560	320	20
<i>C. macrostigmatum</i>	2,560	160	0
<i>A. falcatus</i>	2,560	160	2,560
Monilia	2,560	640	2,560
Aspergillus	2,560	640	2,560
Alternaria	5,120	40	640
Control*	5,120	640	5,120

*Original hemagglutinating titer.

Table IV. Effect of *Chlorella vulgaris* on partial thromboplastin time of human blood

Saline control in seconds	<i>Chlorella vulgaris</i> in seconds
90	120
90	120
80	120
85	115
86	No clot
90	136
110	154
100	120
93	135
84	113
105	130

Table III appear inconsistent when compared with precipitin data, but such a comparison may not be valid in view of the fact that this hemagglutination system probably involves an IgM immunoglobulin.

Hematologic observations

In the course of these serologic investigations, algal extracts were observed to cause hematologic abnormalities. Hemolysis of several species of red cells occurred in vitro with extracts of the algae. In the case of *C. vulgaris* extract, a 1:1,000 dilution produced hemolysis of rabbit, sheep, and human red cells within 2 hours after incubation at 37° C. However no evidence of in vivo hemolysis was observed following intravenous injections in guinea pigs and rabbits. Addition of 0.2 ml. of 1:250 *C. vulgaris* extract to 1.0 ml. human blood prolonged the Lee-White clotting time by a factor of 2. The partial thromboplastin time of human blood was also prolonged after small amounts of algal extract were added to the test system (Table IV). However intravenous injections of *C. vulgaris* extract in rabbits failed to demonstrate clotting abnormalities at 2, 5, and 24 hours after the injections.

DISCUSSION

Immunologic relationships among the green algae as well as between these algae and other allergens were examined in diverse antigen-antibody systems. Precipitating antibodies studied by the double-gel-diffusion system and direct PCA experiments revealed relatively few cross-reactions. On the other hand, PCA and hemagglutination-inhibition experiments demonstrated an increased number of immunologic relationships among the green algae, as well as between algae and the fungi, *Alternaria* and *Aspergillus*.

Although cross-reactive patterns were not entirely consistent, several general relationships emerged from these studies. Algal cross-reactivity was especially evident with antiserum prepared against *S. basilensis*. In the case of *C. botryoides* antigen, precipitin reactions were obtained with *C. vulgaris* and *C. pyrenoidosa* antisera, as well as *S. basilensis* antiserum. Gel-diffusion data suggested that the 3 antisera contained antibodies directed against an immunogenic determinant in *C. botryoides*. It is of further interest that another species, *Chlorococcum macrostigmatum*, showed definite evidence of cross-reactivity. Thus among the 6 algae studied, the 2 species of *Chlorococcum* contain cross-reacting determinants. A similar relationship between *Chlorococcum macrostigmatum* and *Tetracystis* was previously reported by Brown and Lester.¹³

The absence of reverse cross-reactivity was observed in both gel-diffusion and direct PCA experiments. This phenomenon, as reported in other systems, has been attributed to intramolecular differences in the spacing and accessibility of the specific end group as well as charged groups contiguous to the end group.¹⁴

It should be noted that cross-reactivity to fungal antigens was only observed with inhibition techniques and that the incidence of these positive reactions was variable. Data obtained from 79 atopic patients previously skin tested³ failed to establish any significant patterns between algal and fungal antigens. Consideration of clinical results and direct immunologic data obtained in the present study suggest that if cross-reactivity does exist with the fungal antigens tested, it must be of low magnitude. However, the ultimate significance of the fungal cross-reactions noted in inhibition experiments must await immunologic studies with purified antigens.

Neither preliminary clinical studies nor the present investigation have established a definite causal role for green algae in human respiratory allergies. However, it is well known that these organisms are disseminated widely in water, air, and soil and that they are able to induce allergic symptoms under laboratory conditions. The relatively high index of their immunogenicity in animals provides further evidence that algae may contain highly allergenic properties.

Previous human feeding experiments with various strains of green algae had demonstrated that toxic effects of these organisms were mild and limited to the gastrointestinal tract.¹⁵ However, the in vitro hemolytic and anticoagulant properties of *C. vulgaris* encountered in this study suggest that parenteral administration of algal substances in human beings should be limited until these phenomena are investigated more thoroughly.

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