STUDIES ON OPSONINS IN Q FEVER

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A quantitative method for determination of opsonins for *Brucella* has been described (1). Active brucellosis is associated with an increase in blood opsonin, in certain cases to more than 10 million times normal (2). The principles of determination of opsonin for *Brucella* have now been applied to determination of opsonins for rickettsiae.

The experiments have been concerned with the following factors in phagocytosis of elementary bodies of *Coxiella burnetii:* (*a*) Variability of measurement of phagocytosis. (*b*) Phagocytosis in blood of normal human subjects. (*c*) Phagocytosis in blood of vaccinated human subjects. (*d*) Definition of a unit of opsonin. (*e*) Phagocytosis in blood of "normal" subjects living where Q fever is endemic. (*f*) Opsonin titers in Q fever patients; comparison of opsonin titres of serum and heparinized plasma of Q fever patients. (*g*) Comparison of opsonin and complement fixation tests. (*h*) Changes in opsonin activity of stored plasma.

These studies disclosed that the underlying principles affecting phagocytosis of rickettsiae are identical with those observed with bacteria such as *Brucella.* Significant differences in opsonin titer were found between a group of people free of contact with *C. burnetii* and groups immunized by vaccination or by living where Q fever was endemic. During Q fever the opsonin titer may increase many million times. In certain instances the opsonin test was more than 10,000 times as sensitive as the complement fixation test. Sources of error in testing for opsonin were also disclosed.

Method

In general, the determination of opsonin for *C. burnetii* resembles that for *Brucella* (2). It differs only in standardization of rickettsial suspensions and in the stain for visualizing intracellular rickettsiae. To 0.1 ml. of heparinized blood or of equal parts of washed blood cells and diluted plasma was added 0.1 ml. of a standardized rickettsial suspension. This mixture was agitated by rotation for 30 minutes at 37°C. (1). Smears on glass slides warmed to facilitate spreading were stained with Macchiavello stain after drying (1). The elementary bodies of *C. burnetii* stained ruby red and the nuclei blue. The percentage of polymorphonuclear neutrophils containing elementary bodies was determined by examining 50 neutrophils.

Standardization of Rickettsial Suspensions.—The AD strain of *C. burnetii* was employed

in these studies. A suspension of *C. burnetii* was injected into 6- to 9-day chick embryo yolk sacs, which were harvested 8 days later and washed with saline. The resulting suspension, containing approximately 10^{11} LD_{50} doses per cc. for embryonated chick eggs, was concentrated tenfold by centrifugation at 20,000 *g*. To reduce infection hazards in the test, suspensions of phenol-inactivated rickettsiae were used, it having been found that these were phagocytosed quantitatively in the same amounts as infectious rickettsiae. Phenol inactivation was accomplished by treatment with 0.5 per cent phenol for 1 week at 5°C., followed by 2 washes with saline.

The suspension was standardized in terms of the phagocytosis of different concentrations of suspension by leucocytes of guinea pigs, dogs, and man. The minimal concentration of rickettsiae which revealed no less phagocytosis than a higher concentration was selected as the standard concentration. In seventeen tests the same percentage of neutrophils phagocytosed elementary bodies in a 1:5 dilution as in the stock suspension. On the other hand, this percentage was significantly decreased in five of seventeen tests with a 1:10 dilution, and in four of nine blood samples in which phagocytosis was unchanged with 1:5 to 1:10 dilutions, there were decreases with a 1:20 dilution. Therefore, a 1:5 dilution was selected as the standard.

For washing blood cells and diluting plasma, a physiological salt solution containing gelatin was employed. This solution, which has been referred to as Krebs gelatin solution (1), was prepared as follows:

Parts	Solution
50	Knox gelatin P-20, 6 per cent gelatin in 0.9 per cent NaCl
50	0.9 per cent NaCl
4	1.15 per cent KCl
3	1.22 per cent $CaCl_2$ (5 ml. was equivalent to 11 ml. 0.1 N $AgNO_3$
1	2.11 per cent KH_2PO_4
1	3.82 per cent $MgSO_4 \cdot 7\ H_2O$
4.6	5 per cent $NaHCO_3$

RESULTS

Variation in Percentage of Neutrophilic Leucocytes Which Phagocytosed Elementary Bodies of C. burnetii under Standardized Conditions.—It was established that optimal phagocytosis occurred with heparinized blood continuously agitated at 37°C. for 30 minutes. Phagocytosis of *C. burnetii* was most active in neutrophilic leucocytes just as was that of *Brucella* (1). Therefore, a series of experiments was performed to determine variability of reduplicated tests under these optimal conditions. Five samples of blood were selected because 100 per cent of their neutrophils showed phagocytosis of rickettsiae. Each of these samples was divided into 10 aliquots and tested. In the series, 2554 neutrophils were examined and 2522 had phagocytosed rickettsiae, a percentage of 98.7, the same amount as observed with *Br. suis* PSIII under similar conditions (1). Statistical analysis of the data by Dr. Clifford Maloney (Table I) provided a calculation of the probable incidence of phagocytosis percentage values to be expected when different numbers of neutrophils were examined. The table shows that in less than 0.5 per cent of the tests less than 94 per cent of the neutrophils would contain ricketssiae. In other words, if

phagocytosis by less than 94 per cent of the neutrophils were considered as a negative reaction, that arbitrary limit could lead to an incorrect interpretation of less than 0.5 per cent of the positive tests. This analysis determined the range of positive phagocytic reactions accompanying a sufficient amount of opsonin in the plasma. The upper limit of negative phagocytic reactions has

TABLE I

Probable Incidence of Neutrophils with Phagocytosed Rickettsia in Tests with Various Cell Counts When the Population of Phagocytic Neutrophils Had a Mean of 98.7 Per Cent

No. of neutrophils without rickettsiae	No. of neutrophils examined							
	35		40		45		49	
	Percentage of phagocytic neutrophils and probable incidence							
	Phagocytosis	Test incidence	Phagocytosis	Test incidence	Phagocytosis	Test incidence	Phagocytosis	Test incidence
	per cent		*per cent*		*per cent*		*per cent*	
0	100	0.65	100	0.612	100	0.577	100	0.55
1	97	0.925	98	0.906	98	0.887	98	0.87
2	94	0.988	95	0.983	95	0.967	96	0.969
3	92	0.998	93	0.997	93	0.996	94	0.995
4	89	0.999	90	0.999	91	0.999	92	0.999

TABLE II

Per Cent of Individuals in Different Populations with Various Percentages of Neutrophils Phagocytosing C. burnetii

Group	Total No. of subjects	Percentages of subjects exhibiting various degrees of phagocytosis of *C. burnetii* by neutrophils				Mean percentage of positive neutrophils
		Percentage of neutrophils containing C. burnetii				
		0–42	42–80	82–92	94–100	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Normal Camp Detrick	98	50	43	6	1	43.4
Immunized Camp Detrick	145	16	38	16	30	70.5
"Normal" Los Angeles	101	13	38	18	31	71.6

been based upon the phagocytic responses in the presumably normal human blood described below.

Phagocytosis of C. burnetii in Normal Human Blood.—Tests to determine the percentage of neutrophils which phagocytosed *C. burnetii* were performed in 98 individuals at Camp Detrick, Frederick, Maryland, who had no history of contact with this pathogen. A summary is recorded in Table II. Only one subject (or 1 per cent of the test group) showed phagocytosis of *C. burnetii*

by 94 per cent of neutrophils, whereas 50 per cent of the subjects showed phagocytosis by less than 40 per cent of neutrophils. If phagocytosis of *C. burnetii* by 94 per cent of neutrophils were considered abnormal or above normal, this reaction would be expected to occur in only 1 per cent of the normal population, and if considered a positive test it would lead to error in diagnosis in only 1 per cent of normal subjects. These findings and the data of Table I establish the range of positive and negative tests for opsonins of *C. burnetii.*

Phagocytosis of C. burnetii in Blood of Individuals Immunized by Vaccine.—Adult human males and females were injected subcutaneously three times at weekly intervals with 1 ml. of a phenol-inactivated suspension of *C. burnetii* in chick embryo yolk sac. This material was prepared and partially purified by the Rocky Mountain Spotted Fever Laboratory, Hamilton, Montana. Table II summarizes the results of the opsonin tests. Of 145 subjects tested, 30 per cent showed phagocytosis by 94 to 100 per cent of neutrophils, and only 16 per cent by less than 42 per cent of neutrophils, thus revealing considerable difference in phagocytosis of *C. burnetii* by neutrophils of normal and immunized people.

Definition of a Unit of Opsonin for C. burnetii.—Since the error involved in distinguishing a normal from an immune reaction was not more than 1 per cent, a unit of opsonin for *C. burnetii* was defined as that quantity of opsonin which under standardized conditions caused phagocytosis of *C. burnetii* by 94 to 100 per cent of neutrophils. Such a unit of opsonin might occur in the plasma of 1 per cent of a normal population and of 30 per cent of an immunized population.

Phagocytosis of C. burnetii in Blood of Normal Individuals in an Area Where Q Fever Was Endemic.—The suspensions employed for phagocytic studies and immunization contained elementary bodies of *C. burnetii* and proteins of chick embryo infected yolk sac. The difference in phagocytosis in the immunized and normal populations might have been due to opsonins for chick embryo proteins common to both antigens. If these antigens coated the elementary bodies, the opsonins might have stimulated phagocytosis of the elementary bodies. To exclude chick embryo yolk sac as a factor in phagocytosis of *C. burnetii*, tests were performed in people living where Q fever was endemic and also in subjects ill with or recovered from Q fever.

A group of 101 people was studied in the "normal" population of the city of Los Angeles, including 52 applicants for Civil Service positions, 11 patients in the cardiac disease clinic, 21 in the venereal disease clinic, 1 food handler, and 16 office personnel of the city Departnent of Health.[1] None had symptoms of acute infection or was suspected of having any febrile illness. The results

[1] These subjects were made available to us through the courtesy of Dr. John Chapman, Department of Health, City of Los Angeles.

are presented in a scatter chart, Fig. 1, and summarized in Table II. Fig. 1 shows the incidence of percentages of phagocytosis in the normal and immunized populations of Camp Detrick and the "normal" population of Los Angeles. The "normal" Los Angeles population resembled the immunized Camp Detrick group in percentage of neutrophils which phagocytosed *C.*

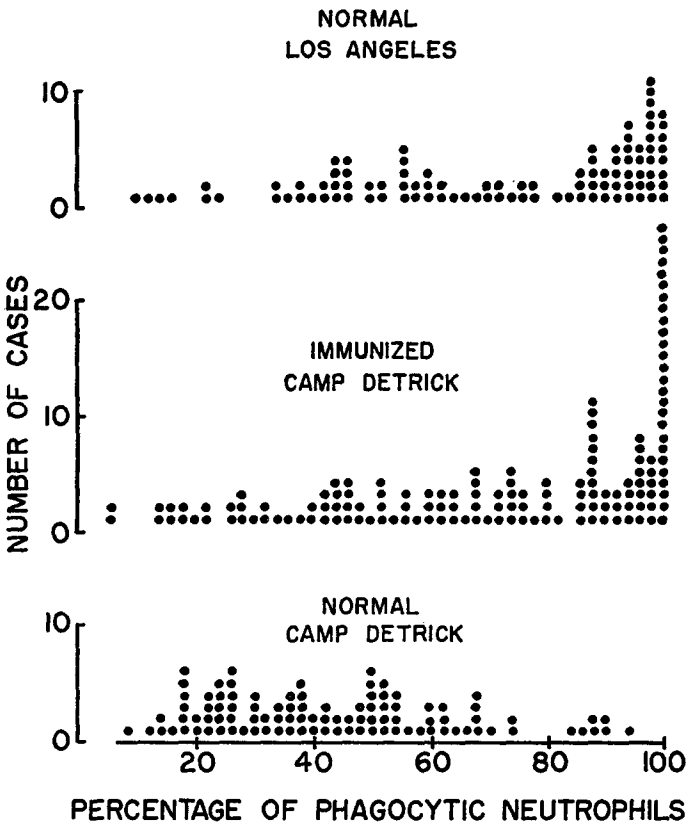

FIG. 1. Percentage phagocytosis of *C. burnetii* by neutrophils. Comparison of blood samples of various populations.

burnetii, and both differed from the normal Camp Detrick group. For comparison of the three populations, the data have been divided into groups according to the percentage of neutrophils phagocytosing *C. burnetii*. If 1 unit of opsonin was considered the maximal normal reaction, then 1 per cent of the normal Camp Detrick population, 30 per cent of the immunized Camp Detrick population, and 31 per cent of the "normal" Los Angeles group reached this limit.

Because opsonins of certain normal individuals might have increased with-

out becoming as high as 1 unit, lower ranges of opsonin activity were studied in the three populations.

The results, including the original standard of 94 per cent for comparison, were as follows:—

Phagocytosis limit	Percentage of subjects at or above limit		
	Camp Detrick normal	Camp Detrick immunized	Los Angeles "normal"
per cent			
94	1	30	31
80	7	49	49
60	22	68	66

There were no significant differences between the Camp Detrick immunized and the Los Angeles "normal" groups at any of the levels listed, although the increase in blood opsonin in the Los Angeles group could not have been influenced by the yolk sac vehicle of the antigen and was therefore related to *C. burnetii*. Comparisons of these groups to the Camp Detrick normal group at the 80 per cent phagocytosis level revealed that 42 per cent of each showed definite evidence of reaction to *C. burnetii*, and approximately the same departure from normal was found at the 60 per cent phagocytosis level. These data indicate that individuals in Los Angeles, where Q fever is endemic, have had considerable exposure to *C. burnetii*, and that at least 42 per cent have reacted immunologically without recognized illness.

Opsonin Titers for C. burnetii in Blood of Patients with Q Fever.—Table III presents data on 11 subjects ill with or recovered from Q fever. The opsonin titer is defined as the number of opsonin units in 0.1 ml. of plasma employed in the test. This titer was determined by the lowest concentration of plasma in which 94 to 100 per cent of neutrophils phagocytosed elementary bodies. The opsonin titer was, therefore, the reciprocal of the lowest plasma concentration in which 94 to 100 per cent of neutrophils phagocytosed *C. burnetii*. These studies compared the opsonin titer of plasma and serum as tested with the patient's blood cells. The plasma and serum were stored at 5°C. and tested a month later with washed cells from group O blood. The results are included in Table III. It may be seen that in recovered cases, C. W., L. T., R. O. G., A. H., and R. B., opsonin titer varied from 10 to 10^2. In subjects ill with the disease, titers ranged from 10^3 to greater than 10^7. The subjects ill with the disease were ambulatory except O. F., who was confined to her home, and C. L. J., who was a hospital patient.[2] These subjects demonstrated that opsonin titer for *C. burnetii* may increase many million times during

[2] We are indebted to Dr. John Chapman of the Department of Health, City of Los Angeles, Dr. Mary B. Dale, and Dr. Roy Gilbert, County of Los Angeles Health Department for making possible the study of these subjects.

illness with Q fever, and that serum gave essentially the same results as heparinized plasma. However, when O blood cells were used with serum or plasma from heterologous donors, the very high titers observed with homologous cells were lowered somewhat. Since a month elapsed between the studies with homologous and heterologous cells, the difference may have been due to deterioration of the opsonin during storage at 5°C. Experiments bearing on this point will be described below.

TABLE III

Opsonin Titers of Individuals with Q Fever

Subject	Date of test	Onset of illness	Opsonin titer				Complement fixation titer	
			Homologous cells		Heterologous cells			
			Plasma	Serum	Plasma	Serum	Plasma	Serum
C. F.*	3/28/50	2/50	10	10				80
L. T.*	3/11/50	4/50	10	10				20
R. O. G.*	7/27/50	1946	10		10			
A. H.*	7/24/50	5/50	10^2	10^2	10	<10	128	128
R. B.*	7/25/50	5/50	10	10		10	16	16
J. D.	7/21/50	1948	$>10^7$	$>10^7$	10^4	10^4	1024	1024
O. F.	7/25/50	1948	$>10^7$	$>10^7$	10^4	$<10^4$	512	512
W. L. S.	7/26/50	3/50	10^3	10^3	$<10^2$	10^2	64	64
M. V.	7/26/50	12/49	10^3	10^3	$<10^2$	10^2	64	64
W. T.	7/26/50	2/50	10^4	10^4	$<10^2$	$<10^2$	128	128
C. L. J.	7/27/50	6/50	10^4	10^4	$<10^2$	10^2		

* Clinically recovered.

Comparison of Opsonin and Complement Fixation Tests.—From the few observations in Table III, it appeared that the opsonin titer might increase as much as 10,000 times more than the complement fixation titer in subjects ill with Q fever. Complement fixation tests performed simultaneously with the phagocytosis tests in the blood of the 101 "normal" Los Angeles individuals described above, and in 90 of the immunized subjects of Camp Detrick, are recorded in Fig. 2, which compares the percentage of neutrophils which phagocytosed *C. burnetii* with the complement fixation titer of the serum in the same individuals. In the Los Angeles "normal" group, 6 complement fixation titers were between 1:8 and 1:32, the other 95 negative. In contrast, 21 of 90 immunized Camp Detrick individuals had complement fixation titers between 1:10 and 1:20. Furthermore, all 6 of the Los Angeles group positive complement fixation tests coincided with phagocytosis of *C. burnetii* by 92 to 100 per cent of neutrophils, whereas the immunized Camp Detrick group showed no relation between complement fixation reaction and phagocytosis.

The lack of intergroup correlation between the complement fixation titers

and the opsonin titers may be due to the difference in methods of immunization of the two populations. Since Q fever was endemic in the Los Angeles area, contact with the agent was probably specific, but the Camp Detrick group was immunized with yolk sac membrane of chick embryo infected with

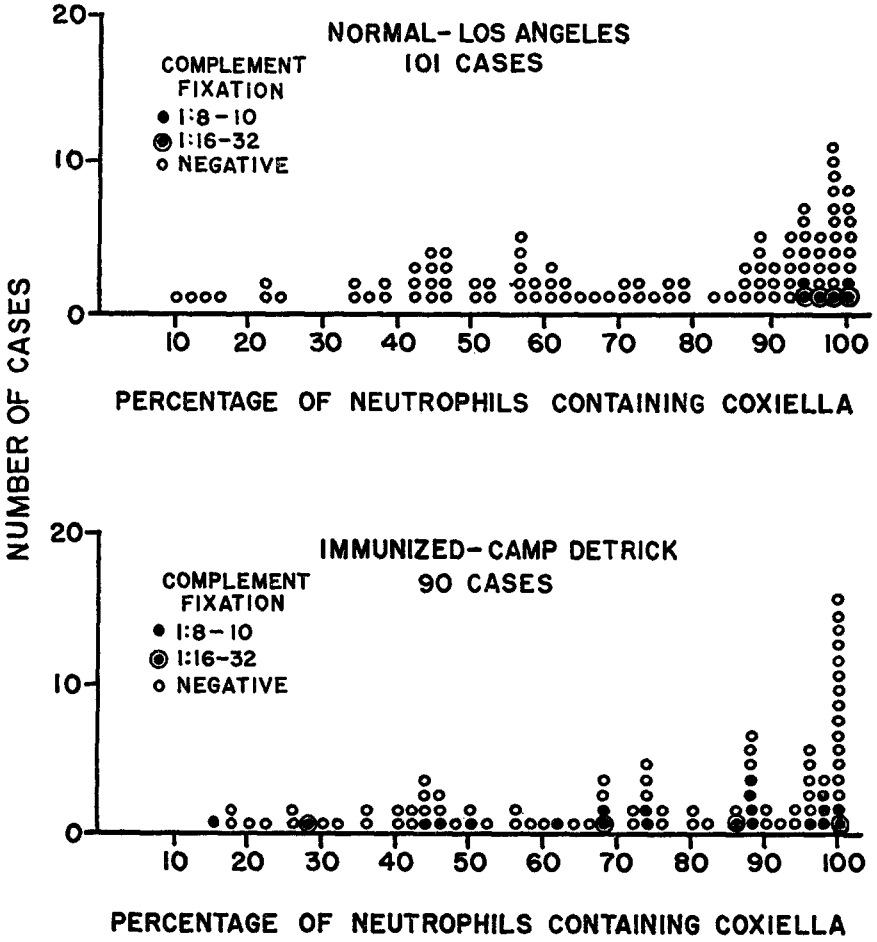

FIG. 2. Comparison of percentage phagocytosis of *C. burnetii* by neutrophils with complement fixation of blood samples of various populations.

C. burnetii. Complement fixation might have occurred in reactions with the inflamed yolk sac antigen as well as with the specific *C. burnetii* antigen. If the complement fixation test did not distinguish between the two reactions, the results might not be specific for either antigen. It is noteworthy that the opsonin test appeared to detect seven times as many cases of immunization

or contact in the Los Angeles population as did the complement fixation test, the relative percentages being approximately 42 and 6.

Changes in Opsonin Titer during Storage of Plasma.—It was mentioned earlier that an observed difference in opsonin titers of homologous and heterologous blood cells might have been due to a loss in opsonin activity during storage of plasma or serum 1 month at 5°C. The plasma of two patients has been studied for opsonin titer during storage at 5°C. for 4.5 months. Opsonin titers were determined with group O cells from the same donor. The opsonin titers were 10^7, 10^4, 10^3, and 10^3 in one sample at 0, 38, 84, and 138 days respectively, and 10^6, 10^3, and 10^2 in another sample at intervals of 0, 32, and 138 days after bleeding. Storage of plasma at 5°C. for 4.5 months therefore decreased the opsonin titer for *C. burnetii*.

DISCUSSION

The evidence cited above demonstrated that phagocytosis of elementary bodies of *C. burnetii* was governed by the same factors affecting phagocytosis of bacteria such as *Br. suis*. These factors were time of exposure, temperature, agitation, number of organisms, type of phagocytic leucocyte, and the anticoagulant. Since there was no difference in the rate of phagocytosis of living and dead agents, the methods for determining opsonins were freed of infection hazards by employing dead agents.

Quantitation of plasma opsonin for *C. burnetii* has several sources of error. Storage of serum or heparinized plasma at 5°C. lowered the opsonin titer. Unpublished experiments in guinea pigs demonstrated that injection of normal yolk sac slightly increased opsonin for *C. burnetii*. Plasma of vaccine-immunized subjects at Camp Detrick showed no relation between opsonin and complement fixation titer. Humans vaccinated with antigens harvested from chick embryo may produce opsonins for antigens of chick embryo unrelated to *C. burnetii*. While non-specific reactions were possible in the population which had been immunized by vaccine at Camp Detrick, the increased opsonins and complement fixation titer in the Los Angeles group could not have been due to non-specific reactions. The latter subjects had not been immunized with egg vaccine. Individuals with Q fever and those recovered from this disease had not been treated with vaccine.

The combined data demonstrated that the opsonin test for *C. burnetii* offers a convenient, inexpensive, and easy test which is applicable to both epidemiological and diagnostic purposes in the study of Q fever. Application of this test to other rickettsial diseases is worthy of investigation.

SUMMARY AND CONCLUSIONS

A method was developed for the quantitative determination of opsonins for *C. burnetii*.

An opsonin unit for *C. burnetii* was defined as that quantity of opsonin which, under standardized conditions, causes phagocytosis of elementary bodies of *C. burnetii* by 94 to 100 per cent of polymorphonuclear neutrophils. The opsonin titer represents the number of opsonin units in 0.1 ml. of serum or heparinized plasma.

A plasma opsonin titer of 1 occurred in 1 per cent of the people tested at Camp Detrick, Frederick, Maryland, with no known contact with *C. burnetii;* in 30 per cent of the immunized people of Camp Detrick treated by subcutaneous injection of phenol-inactivated *C. burnetii* in chick embryo yolk sac; and in 31 per cent of the people tested at Los Angeles, with no clinical evidence of Q fever.

On a statistical basis, about 42 per cent of the vaccinated people of Camp Detrick and of "normal" Los Angeles people had evidence of increased opsonins in their blood.

Vaccinated individuals of Camp Detrick showed no relation between opsonin and complement fixation titers, but individuals living in Los Angeles, an area endemic for Q fever, revealed positive complement fixation tests only in one-seventh of individuals with opsonin titers of 1. The significance of these differences is discussed.

The opsonin titer of patients with Q fever may increase many million fold, and much more than the complement fixation titer. The opsonin titers of serum and heparinized plasma were identical, and both decreased with storage at 5°C.

The opsonin titer for elementary bodies of *C. burnetii* grown in chick embryo yolk sac may be used as an epidemiological and diagnostic indicator for the distribution of the agent causing Q fever.

BIBLIOGRAPHY

1. Victor, J., Pollack, A. D., Raymond, R., and Valliant, J., *J. Bact.*, in press.
2. Victor, J., Pollack, A. D., Valliant, J., and Raymond, R., *J. Am. Med. Assn.*, in press.