IMMUNOLOGIC STUDIES IN ONCHOCERCIASIS AND BANCROFTIAN FILARIASIS

I. Intracutaneous Tests with Antigens Extracted from *Onchocerca* and *Dirofilaria**

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Numerous reports concerning intracutaneous tests (IC) in filarial diseases have appeared during the past thirty years. This subject was reviewed extensively in a recent paper by Kagan.[1] Table 1 summarizes some of the reported results from tests of infected and normal subjects.

Three facts emerge from a review of these reports: (1) There is a wide range of variation in the results obtained in both the test subjects and the controls. (2) The variations derive partly from differences in technique of antigen preparation, in the amount and strength of the antigens used, in the criteria of positivity employed by different investigators, and probably from real differences in the populations being tested. (3) No attempt has been made to evaluate IC tests in bancroftian filariasis with an antigen prepared from *Onchocerca*.

As pointed out by Kagan,[1] the lack of adequate standardization of methods and techniques has been the most important single factor contributing to the present confusion surrounding the value of IC tests in filarial diseases. The present study, therefore, represents an effort to evaluate intradermal tests by employing standardized antigens and techniques, as recommended in a technical report of the World Health Organization.[2] It also attempts to determine the relative reactivity to antigens prepared from saline and lipid-free extracts, and to compare the results with reference to onchocerciasis and filariasis independently by performing tests in widely separated endemic areas. Possible correlations between the results of intracutaneous tests and the diagnostic signs of these infections were also investigated.

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MATERIALS AND METHODS

Antigens

Antigens were prepared from *Onchocerca volvulus* and *Dirofilaria immitis* as follows:

Onchocerca nodules removed surgically from infected persons in Guatemala were frozen immediately, transferred to dry ice, and sent by air express to Los Angeles where they were held at −20°C. The nodules were thawed, opened with a scalpel and the individual worms were removed manually with small forceps, using a head-mounted magnifying lens. The worms were then washed in buffered saline, freeze-dried and kept at −20°C.

The *Dirofilaria* worms were collected from the hearts of dogs in Tahiti, frozen immediately at −20°C, and shipped in the frozen state to Los Angeles where they were washed in buffered saline and freeze-dried.

The freeze-dried worms were disintegrated in a tissue grinder and extracted in saline, with or without prior delipidization, as described by Rieber *et al.*[3] Melcher extracts of *Dirofilaria* were kindly supplied for these studies by Dr. I. G. Kagan. Nitrogen determinations were made by the micro-Kjeldahl method, before and after heating. These determinations correlated well with the protein content measured by the method of Lowry *et al.*[4]

The antigens were sterilized by heating at 65°C for 5 hours and tested routinely for aerobic and anaerobic bacteria by culture methods. Antigens were diluted and preserved in a 1:10,000 merthiolate-saline solution so as to contain equivalent N concentrations at various levels for the purpose of reciprocal comparison.[5, 6] The antigenic extracts thus prepared for testing are shown in Table 2.

TABLE 1

Summary of intracutaneous tests reported in previous publications

Antigen used	Persons with	No. reports	Range in % positive	
			Patients	Controls
Onchocerca	Onchocerciasis	6	42–100	2–18
	Filariasis	0	—	—
Dirofilaria	Onchocerciasis	7	70–96	35–55
	Filariasis	35	46–100	4–19

TABLE 2

Nitrogen content of antigens employed

Antigen	Nitrogen content in gamma/ml			
Saline extract of *Onchocerca*	.16	.40	.80	4.0
Saline extract of *Dirofilaria*	.21	.50	1.1	3.1
Lipid-free extract of *Onchocerca*	.28	—	1.4	—
Melcher extract of *Dirofilaria*	.26	—	1.3	2.8

Techniques

The volar surface of each arm was swabbed with alcohol and allowed to dry. With a 27-gauge platinum needle attached to a 0.25-ml tuberculin syringe, 0.05 ml of antigen was injected intracutaneously, care being taken to avoid overlapping whenever several tests were performed on the same subject at the same time. An equal amount of 1:10,000 merthiolate-saline was injected as control. Needles were flamed between injections. Reactions were recorded after 15 minutes, the area of the wheal being determined by circling with a ballpoint pen and transferring the ink marks onto alcohol-moist paper which was then measured with the cellophane template described by Kagan *et al.*[5] The injection of 0.05 ml of merthiolated saline produced wheals which ranged between 0.1 and 0.8 cm^2 in all of the test and control groups (Fig. 1), and, therefore, only the reactions with an area of 1.0 cm^2 or more were considered positive.

Three hundred and eight indigenous persons were tested in Guatemala, 385 were tested in Samoa, and 34 controls were tested from non-endemic areas.

Populations

a) Studies on onchocerciasis were conducted at Finca Mocá, Suchitepéquez, Guatemala, which has a stable population of approximately 1,000 laborers.

All subjects were examined for the presence of nodules and asked whether any nodule had been previously removed at any time. Two skin biopsies, one from the shoulder and one from the calf, were also obtained from each person prior to the application of skin tests. Thus, four groups were obtained:

1) Persons having one or more *Onchocerca* nodules and at least one positive biopsy at the time of examination (159 subjects).

2) Persons having one or more *Onchocerca* nodules but no microfilariae in the skin biopsies (24 subjects).

3) Those having no nodules or microfilariae found at time of IC tests, but having had nodules removed sometime previously (87 subjects).

4) Those having no nodules or microfilariae and no history of having had nodules removed at any time in the past (38 subjects).

For the purpose of this study, groups 1 and 2 were considered "positive" for onchocerciasis, while groups 3 and 4 were considered "negative."

b) Studies on filariasis were conducted in three villages of the Island of Tutuila, American Samoa, whose population had been surveyed by physical and blood examinations 6 months previously and had then received treatment with diethylcarbamazine.[7]

Prior to the application of the IC tests, all subjects were re-examined for microfilariae, thus providing three groups:

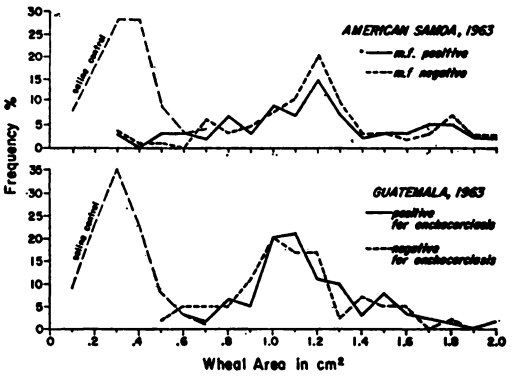

FIGURE 1. Frequency distribution of wheal area in reactions to *Onchocerca* antigen containing 4 gamma N/ml.

1) Persons still positive (microfilaremia) 6 months after a course of therapy (60 subjects).

2) Those who became negative during the 6 months following therapy (83 subjects).

3) Those who were earlier, and had remained, negative (242 subjects).

For the purpose of this study, groups 1 and 2 were considered "positive" for filariasis while group 3 was considered "negative."

c) Controls included healthy persons, some of whom did, and others who did not, harbor intestinal nematodes, neither group having resided in endemic areas of onchocerciasis or filariasis.

RESULTS AND DISCUSSION

The cutaneous reactivity of persons with demonstrable onchocerciasis or filariasis to the control solution of merthiolated saline did not differ significantly from that of normal adults residing in the U.S.A. or non-infected residents of the same endemic area. Likewise, children and adults reacted similarly to the control solution. It would appear, therefore, that neither age nor the presence of active filarial infections influenced the cutaneous reactivity of these endemic populations to the injection of merthiolated saline alone. This fact is demonstrated by comparing the two saline control curves shown in Figure 1. This figure also shows the frequency distribution of the wheal areas obtained from currently infected ("positive") and currently (uninfected) "negative" persons in the two endemic areas, for a given antigen concentration. A similar general pattern of curves was obtained when the data from the other antigen concentrations were tabulated in the same fashion. It was apparent that the mean wheal areas produced by each N concentration of the three extracts did not vary significantly between infected and uninfected persons or between the two endemic populations.

Reactivity to Antigens Having Different Concentrations of Nitrogen

Table 3 summarizes the results obtained with several concentrations of *Onchocerca* and *Diro-*

TABLE 3

Results of intracutaneous tests with Onchocerca *and* Dirofilaria *antigens of various concentrations, measured in terms of nitrogen content, on subjects with and without filarial infections in Guatemala and Samoa*

Type of subjects*	No. positive/no. tested (and % positive) with antigens of *Onchocerca* (O) or *Dirofilaria* (D) in saline containing												
	Whole worm extract & μg N/ml in amts. of								Lipid free extracts & μg N/ml in amts. of				
	0.16 O	0.21 D	0.40 O	0.50 D	0.80 O	1.1 D	4.0 O	3.1 D	0.28 O	0.26 DM†	1.4 O	1.3 DM	2.8 DM
Guatemala													
Infected	0/57	4/60 (6)	—	—	37/60 (61)	17/62 (27)	51/62 (82)	31/38 (81)	—	—	11/39 (28)	2/37 (5)	20/37 (54)
Non-infected	0/33	2/38 (5)	—	—	11/40 (27)	12/44 (27)	28/41 (68)	12/16 (75)	—	—	9/21 (42)	1/21 (4)	10/16 (62)
Samoa													
Infected	0/10	0/10	0/16	5/10 (50)	31/76 (40)	30/59 (50)	47/62 (76)	—	0/21	0/21	15/31 (48)	2/32 (6)	—
Non-infected	0/25	0/25	4/15 (26)	12/19 (64)	57/129 (44)	66/115 (57)	79/108 (73)	—	5/56 (8)	6/55 (10)	24/66 (36)	12/63 (19)	—
Controls													
Normal	0/13	0/12	—	—	0/19	0/8	0/15	—	—	—	—	—	0/14
With int. helm.	—	—	—	—	0/19	0/19	0/19	0/19	—	—	—	—	—

* Subjects with nodules or microfilariae in biopsies, in Guatemala, and those with microfilaremia when tested, or before treatment 6 months earlier, in Samoa, were classified as "infected" and all others as "non-infected."

† Melcher extract of *Dirofilaria*.

filaria antigens, extracted by three different methods and adjusted to contain equivalent amounts of N/ml. In the table, the letter "O" identifies the *Onchocerca* antigen, the letter "D" the *Dirofilaria* antigen and the letters "DM" the Melcher extract of *Dirofilaria*. The subjects were grouped as "positive" and "negative" according to the criteria described under "populations."

The data indicated that persons with onchocerciasis or filariasis reacted to both antigens in percentages that were proportional to the antigenic concentration of the extract used, as measured by their nitrogen content. The maximum percentages of reactivity were thus obtained with antigens containing 3 to 4 μg N/ml, which were the highest concentrations used in these studies. Little or no reactivity was obtained with the lowest concentration of antigen, *e.g.*, between .16 and .28 μg N/ml. Lipid-free extracts of the Melcher type showed, in general, less reactivity than did the corresponding concentration of lipid-free *Onchocerca* or saline extracts of *Dirofilaria* or *Onchocerca*. It was apparent, therefore, that a logarithmic relationship exists between the N content of the three antigens tested and their cutaneous reactivity as expressed by the percentage of positive reactions. No positive reactions were encountered among the control groups tested.

The data also indicated that in Guatemala the homologous *Onchocerca* antigen containing .80 μg N/ml was considerably more reactive than was the *Dirofilaria* antigen containing 1.1 μg N/ml. In Samoa, where both antigens were heterologous to *Wuchereria bancrofti*, saline extracts of *Dirofilaria* containing between .50 and 1.1 μg N/ml were more reactive than were the corresponding saline or lipid-free extracts of *Onchocerca*.

At higher concentrations of antigen, *e.g.*, between 3.1 and 4.0 μg N/ml, *Onchocerca* and *Dirofilaria* antigens both produced approximately the same high percentage of reactivity in Guatemala. The *Onchocerca* antigen stimulated a similar percentage of positive tests in Samoa.

Correlations with Diagnostic Signs of Onchocerciasis or Filariasis

The data in Table 3 show that in the endemic area of Guatemala a certain degree of positive correlation was attained between the presence of nodules and/or microfilariae and positive IC reactions to homologous saline extracts of *O. volvulus* containing 0.8 μg N/ml. It can be seen in Table 3 that 61% of the persons with positive findings of onchocerciasis at the time of skin testing exhibited positive IC tests, whereas in the group of persons without currently demonstrable nodules or microfilariae only 27% reacted positively. Tests with heterologous antigen of *D. immitis* did not distinguish between these groups. When *Onchocerca* and *Dirofilaria* antigens were employed at the higher concentration of 3.1 to 4.0 μg N/ml, a greater percentage of reactivity was obtained with both, but at this antigenic level the test showed little ability to distinguish between persons with currently positive diagnostic signs, and those with negative signs.

These findings become more apparent in Table 4, where the subjects were grouped in diagnostic categories: It may be seen that the frequency of positive skin tests in Guatemala increased with the intensity of the exposure or infection, when the *Onchocerca* antigen containing 0.8 μg N/ml was used.

Little difference could be detected among these groups, however, when tested with *Onchocerca* antigen at a concentration of 4.0 μg N/ml or with *Dirofilaria* antigen. As previously shown in Figure 1, the distribution of mean wheal areas of positives and negatives in the two endemic populations also did not differ significantly for the same antigen concentration.

No significant correlation could be found between diagnostic signs of bancroftian filariasis and the results of IC tests in the indigenous population of Samoa, whether *Onchocerca* or *Dirofilaria* antigens were used.

Sex and Age Distribution

The sex of the subjects tested had no apparent influence on either the frequency of positive intracutaneous tests or the size and distribution of the mean wheal areas obtained in the two indigenous populations, regardless of the type of antigen or the N concentration employed.

Table 5 presents the frequencies of positive intracutaneous tests arranged by age-groups.

It is apparent that in Guatemala and Samoa the frequency of positive IC reactions to antigens containing .80 to 1.1 μg N/ml increased with age during the first 15 years of life and remained relatively constant thereafter. *Onchocerca* antigen with an N concentration of 4 μg N/ml elicited

TABLE 4

Frequency of positive reactions to intracutaneous tests by groups tested with saline extracts

Groups*	Antigen	*Onchocerca*				*Dirofilaria*	
	μg N/ml.....	0.8		4.0		1.1	
		No. +/no. exam.	%+	No. +/no. exam.	%+	No. +/no. exam.	%+
Guatemala							
1		33/49	67	46/55	84	17/55	30
2		4/10	40	5/7	71	1/7	14
3		8/25	32	19/29	65	7/33	21
4		3/15	20	9/12	75	5/11	45
Samoa							
1		10/23	43	16/21	76	6/16	37
2		11/31	36	19/27	70	12/25	48
3		39/101	38	69/90	74	52/88	59

* Groups—*Guatemala:* 1, *Onchocerca* nodule +; biopsy +. 2, *Onchocerca* nodule +; biopsy −. 3, *Onchocerca* nodule removed previously. 4, *Onchocerca* nodule −; biopsy −. *Samoa:* 1, mf carrier + 6 months after treatment. 2, mf carrier − 6 months after treatment. 3, mf negative before and after treatment.

TABLE 5

Frequency of positive intracutaneous reactions to saline extracts of Onchocerca *and* Dirofilaria *in two populations arranged by age-groups*

Age groups by yrs	Antigen	Guatemala				Samoa			
		Onchocerca				*Onchocerca*		*Dirofilaria*	
	μg N/ml....	.80		4.0		4.0		1.1	
		No. +/no. exam.	%+	No. +/ no. exam	%+	No.+/no.exam.	%+	No.+/no.exam.	%+
0–5		0/12	0	—		15/18	83	7/19	36
6–10		4/14	28	17/21	77	24/43	55	14/44	32
11–15		6/9	67	14/18	72	21/27	77	17/26	65
16–20		6/11	54	18/22	74	11/12	91	5/8	62
21–40		20/36	55	41/49	81	31/35	88	35/46	76
41+		12/18	67	16/21	69	34/40	85	20/32	65

high percentages of reactions in all age-groups of both populations.

SUMMARY

Intracutaneous tests were performed with saline, lipid-free and Melcher extracts of *Onchocerca* and *Dirofilaria* on persons living in areas endemic for onchocerciasis in Guatemala and bancroftian filariasis in American Samoa. When measured by their N concentration, coupled with standardized methods of extraction, administration and measurement of reactivity, a logarithmic relationship appeared to exist between the wheal area and nitrogen content of injected antigen. Antigens prepared by saline extraction were generally more reactive than were antigens prepared by the other methods.

Concentrations of antigens from 3 to 4 μg N/ml elicited high percentages of positive reactions in persons from endemic areas, while normal controls and persons with intestinal nematodes from non-endemic areas gave negative reactions. Antigens with a strength of 0.8 μg N/ml yielded a lower reactivity, and may be nearer an antigen concentration that will distinguish between currently infected and non-infected persons than the higher concentrations.

Dirofilarial extracts appeared to elicit group

reactions in the two populations while onchocercal extracts elicited more specific reactions in persons infected with *Onchocerca*. The frequency of reactions by age-groups in both populations increased with age and with the nitrogen concentration of the antigen employed. No variations by sex were observed.

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