VACCINATING PROPERTIES OF AVIRULENT DISSOCIATES OF FIVE DIFFERENT STRAINS OF TUBERCLE BACILLI*

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In a period such as the present one, characterized by great advances in chemotherapy, interest in vaccines is at a low ebb. But in the case of tuberculosis, vaccination should probably play a greater part than it has in the experimental assay of new chemical remedies. We have felt that the animals ordinarily used in the laboratory have so little native resistance to the tubercle bacillus that only the most potent drug could be expected to check the course of primary infections with this microorganism. To avoid this dilemma, investigators have frequently resorted to the practice of saturating the tissues with a drug for a period of days or weeks before administering infection. But this procedure has little place in human therapy, and in animals it has engendered false optimism. Bacteriostatic properties may hold the microorganisms in check for days or weeks, but if the animals are allowed to live long enough most drugs are finally eliminated and the tubercle bacilli are then free to multiply and cause disease. We believe that it is much more logical to enhance the low native resistance of guinea-pigs or rabbits to the tubercle bacillus by preliminary vaccination and to test the effect of new remedies during the course of re-infection with a small dose of virulent tubercle bacilli. There are some who will not agree, for they believe that the goal of chemotherapy in human tuberculosis is treatment of the newly discovered, progressive, primary lesion; the chronic lesions, they feel, are too far advanced to benefit greatly. But to them, we would reply that progressive, primary foci that are large enough to be detected in a roentgenogram of the lungs have already become re-infection tuberculosis although endogenous in origin.

From time to time, we used all of the accepted methods of vaccinating animals against virulent tubercle bacilli:

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(1) Living organisms of low virulence for particular hosts, such as human type bacilli in rabbits, or the Friedmann bacillus or the vole bacillus in guinea-pigs.
(2) Spontaneously attenuated mammalian strains like R_1 or B.C.G. which have lost virulence after prolonged cultivation on artificial media.
(3) Cultures of virulent strains killed by heat or chemicals.

All of these will increase resistance to re-infection, prolong life, and with properly selected doses, cause a tuberculosis that is much more chronic than that in primarily infected animals. But the results are rarely uniform in any large series of animals and uniformity of disease is one of the prime requisites in assaying the effect of a new therapeutic agent. Some animals die early with fairly acute lesions, others later with widespread chronic disease, and there are usually some, that have to be sacrificed to terminate the experiment, which show no macroscopic evidence of tuberculosis. Admittedly, much of this variation is due to individual inheritance, but we have never ceased to wonder whether choice of vaccinating agent, interval before re-infection, or other technical considerations may not have been partly responsible.

In our search for a more reliable vaccine, we have turned to the variants produced by bacterial dissociation, because this technic offered a controlled means of obtaining tubercle bacilli of low virulence that would remain stable for years. It was hoped that they might also possess immunizing properties of equal stability. This possibility had to be explored in spite of the experience with certain other bacteria whose low virulent dissociants have yielded low titers of protective antibodies.

Avirulent variants of five different strains of tubercle bacilli that had been grown on artificial media for a period of four years without appreciable change were available for study. The sources of these cultures are shown in Table 1. They include two well-known strains, H_{37} and R_1, and three isolated more recently. They had been kept upon a modified Proskauer and Beck medium* which

*Monopotassium phosphate ..... 5.0 gm.
Asparagin ..... 5.0 gm.
Magnesium sulphate ..... 0.6 gm.
Magnesium citrate ..... 2.5 gm.
Glycerol ..... 20.0 cc.
Make up to 1000 cc. with H_2O, adjust to pH 7.4.

TABLE 1

SOURCES OF CULTURE USED

Strains of microorganisms	*Age of patient*	*Source*
J. H. 6	19 months	Mesenteric lymph nodes, autopsy. Isolated by Steenken, 1937.
R_1		Lung. Isolated by Dr. E. L. Trudeau, 1891.
J. H. 16	3½ years	Pus from flank abscess. Isolated by Steenken, 1937.
H_{37}	19 years	Sputum of patient with chronic pulmonary tuberculosis. Isolated by Dr. E. R. Baldwin, 1905.
H_4	44 years	Sputum of patient with chronic progressive Tb of productive type with cavity. Persistently high Gaffky (v to x). Duration 2 years+. Isolated by Steenken, 1937.

has proved particularly effective in maintaining virulence. All of them exhibited identical cultural characteristics which had undergone no change in the four years since they were first described for publication.[1] Their colony structure, and rate and character of growth still remained the same. The virulence of all of them had been tested repeatedly in animals and never had any of them produced progressive tuberculosis. Intracerebral, intratesticular, and subcutaneous inoculation into guinea-pigs had in all cases resulted in a localized focus of subacute inflammation, with healing by resolution after approximately three weeks. Rapid passage through the testes of a series of 20 guinea-pigs, over a period of six months had not altered their virulence or changed growth characteristics on artificial media.

Experimental procedures

Program of treatment: To compare their efficiency as vaccines, each of these "Ra" dissociates was administered subcutaneously to a group of 25 tuberculin-negative guinea-pigs, each group equally divided according to sex, with most of the animals weighing between 350 and 450 gms. Two weeks later all of these animals with 25 unvaccinated control pigs were given a subcutaneous inoculation of a standard dose of virulent "Rv" dissociant of strain H_{37}. Duration of life and extent of disease were to serve as the bases of comparison. The original plan to permit all of the animals to die was not strictly followed. The six that remained alive for longer than 20 months after re-infection were sacrificed to terminate the experiment.

TABLE 2

	CONTROLS			VACCINATED GROUPS					
				J. H. 6-"Ra"			R_1 "Ra"		
Sex	*Extent Tb*	*Days survival*	*Cause of death*	*Extent Tb*	*Days survival*	*Cause of death*	*Extent Tb*	*Days survival*	*Cause of death*
Females	14	88	Tb	15	158	Tb	12	110	Tb
	15	104	Tb	13	172	Tb	16	131	Tb
	15	123	Tb	16	194	Tb	15	182	Tb
	16	130	Tb	14	194	Tb	13	200	Tb
	16	145	Tb	9	214	Ent.	9	208	Tb
	16	158	Tb	9	215	?	15	267	Tb
	14	162	Tb	15	270	Tb	11	327	?
	16	170	Tb	15	272	Tb	14	337	Tb
	16	176	Tb	16	312	Tb	16	357	Tb
	15	180	Tb	15	382	Tb	14	372	Tb
	14	208	Tb	5	398	Ent.	11	375	Tb
	15	232	Tb	10	399	Pn. Pn.[4]	13	465	Tb
	16	252	Tb	0	617	Killed	12	490	Tb
	16	281	Tb	..	...	...	0	617	Killed
Males	10	88	Tb	6	88	Ent.	14	111	Tb
	9	95	Ent.[1]	3	104	Strep. Pn.[2]	8	112	Strep. Pn.
	14	120	Tb	2	139	Strep. Pn.[2]	16	179	Tb
	12	137	Tb. Snf.	15	174	Tb	15	200	Tb
	16	168	Tb	15	176	Tb	15	265	Tb
	15	176	Tb	16	198	Tb	12	273	Tb
	14	193	Tb	1	205	Strep. Pn.	12	281	Strep. Per.[5]
	16	212	Tb	15	219	Tb	16	300	Tb
	16	292	Tb	16	219	Tb	14	435	Tb
	9	340	?	13	253	Tb	15	493	Tb
	7	383	Ent.	13	264	Strep. Pn.	15	517	Tb
	..	...	...	16	298	Tb	..	...	...

[1] Ent. = Enteritis.
[2] Strep. Pn. = Streptococcus pneumonia.
[3] Snf. = Snuffles.
[4] Pn. Pn. = Pneumococcus pneumonia.
[5] Strep. Per. = Streptococcus peritonitis.

Preparation of vaccines: Each of the 5 vaccines was made from 15-day growths of microorganisms on the Proskauer and Beck medium. The bacteria were removed from the surface of the culture fluid and triturated in a sterile mortar with physiological

TABLE 2

VACCINATED GROUPS

J. H. 16 "Ra"			H_{57} – "Ra"			H_4 – "Ra"		
Extent Tb	*Days survival*	*Cause of death*	*Extent Tb*	*Days survival*	*Cause of death*	*Extent Tb*	*Days survival*	*Cause of death*
8	190	Pn. Pn.	2	201	Strep. Pn.	10	172	Snf.[a]
3	261	Strep. Pn.	14	212	Tb	9	251	Ent.
15	274	Tb	7	217	Strep. Pn.	11	302	Strep. Pn.
15	306	Tb	2	281	Strep. Pn.	11	305	Strep. Pn.
14	309	Tb	13	373	Tb	8	322	Strep. Pn.
11	315	Strep. Pn.	14	414	Tb	15	356	Tb
15	327	Tb	13	454	Tb	12	363	Tb
16	349	Tb	15	471	Tb	15	439	Tb
14	368	Tb	15	515	Tb	15	452	Tb
14	378	Tb	8	568	?	15	468	Tb
16	429	Tb	9	570	Ent.	7	484	?
6	500	Ent.	4	617	Killed	8	486	Strep. Pn.
3	576	Ent.	7	617	Killed	4	512	?
..	...	...	..	...	...	..	...	...
1	82	Strep. Pn.	12	198	Tb	3	175	Ent.
3	88	Pn. Pn.	10	200	Ent.	10	195	Ent.
9	155	Tb	2	257	Strep. Pn.	9	320	Strep. Per.
7	205	Strep. Pn.	11	300	Snf.	9	324	Strep. Per.
9	212	Ent.	14	333	Tb	4	327	Strep. Pn.
6	214	Strep. Pn.	13	372	Tb	8	409	Ent.
10	218	Ent.	11	392	Tb	16	441	Tb
14	249	Tb	11	393	Tb	15	479	Tb
13	298	Tb	12	410	Tb	11	479	Ent.
11	312	Strep. Pn.	9	569	?	10	558	Ent.
15	383	Tb	6	594	?	4	617	Killed
9	528	?	..	...	Lost	2	617	Killed

salt solution until a uniform suspension was obtained. A small portion was then removed to determine the concentration by evaporating the fluid upon a water-bath and noting the weight of the dried organisms. On the basis of this information, the balance of the suspension was then diluted with physiological salt solution so that each 0.5 cc. contained 2.5 mg. of tubercle bacilli.

Vaccination was effected by 3 subcutaneous injections of 2.5 mg., administered every other day, a total of 7.5 mg. Two weeks after the last injection, all animals were skin-tested intracutaneously with 5 per cent old tuberculin. All reacted vigorously with erythema and induration that persisted for 48 hours.

The test inoculation with virulent tubercle bacilli was administered 2 weeks later by subcutaneous injection of approximately 50,000 bacilli* of the virulent "Rv" variant of strain H_{37}. The 25 unvaccinated were injected at the same time with the same dose.

As death occurred, the animals were autopsied and the extent of disease in each was assessed. For purposes of record and comparison, the involvement of the spleen, liver, lungs, and lymph nodes was individually assigned a value proportional to the extent and the severity of the tuberculosis. The maximum rating of 4 in any organ was used to indicate widespread caseous disease diagnosed by gross inspection. The maximum value of 16 for the animal as a whole signified advanced generalized tuberculosis.

The severity of the disease in the body as a whole, the duration of life, and the cause of death for each animal in the experiment are summarized in Table 2. This table covers the infection control group and the five different vaccinated groups. Tables 3, 4, and 5 summarize these results.

Table 3 shows the average period of survival for the six different groups and distributes their members by cause of death. The figures demonstrate that all five of these "Ra" dissociates of tubercle bacilli confer appreciable degrees of protection against re-infection. They also suggest that the vaccines from two of the strains, H_{37} and H_4, are more potent than those from the other three. While 36 per cent of all animals of the vaccinated groups died from intercurrent infections, most of these deaths occurred long after re-infection (Table 2) and, hence, have not materially invalidated the experiment. The animals had sufficient resistance to tuberculosis so that they survived to succumb to other infections of accidental origin.

Table 4 records the duration of life and the extent of disease in the first and last member of each group to die of tuberculosis. The extremes of survival are appreciably longer in the vaccinated

* This number has been computed on the assumption that 1 mg. of dry organisms contains 300,000,000 tubercle bacilli.

than in the non-vaccinated controls, but there is considerable variation from one vaccinated group to another. The point of importance is the fact that the *extent* of the disease is essentially the same

TABLE 3

Groups	AVERAGE NUMBER OF DAYS OF LIFE					
	No. of animals	*Animals dying of tuberculosis*	*No. of animals*	*Animals dying of intercurrent disease*	*No. of animals*	*All animals in the group*
Controls	22	173 days	3	273 days	25	185 days
J. H. 6	15	233 days	9	224 days	24	229 days
R_1	21	298 days	3	240 days	24	291 days
J. H. 16	12	319 days	13	285 days	25	301 days
H_{37}	12	378 days	10	376 days	22	377 days
H_4	7	428 days	16	351 days	23	374 days

in the last as in the first member to die within each group. The animals that died of tuberculosis all had a generalized infection. It is inferred, but it is not obvious, that a general dissemination had occurred early in all cases and had merely lasted longer in some of the animals.

TABLE 4

Groups	FIRST AND LAST ANIMAL TO DIE OF TUBERCULOSIS						
	Days of life		*Rating*				
	First	*Last*	*Lungs*	*Liver*	*Spleen*	*Nodes*	*General rating*
Controls	88		3	4	3	4	14
		292	4	4	4	4	16
J. H. 6	158		4	3	4	4	15
		382	3	4	4	4	15
R_1	110		2	2	4	4	12
		517	3	4	4	4	15
J. H. 16	249		3	4	4	3	14
		429	4	4	4	4	16
H_{37}	212		4	2	4	4	14
		515	3	4	4	4	15
H_4	356		3	4	4	4	15
		479	4	3	4	4	15

Table 5 summarizes the observations on the six survivors killed, on the 617th day after re-infection, to terminate the experiment. Two of these animals presented no gross evidence of tuberculosis; the other four showed relatively little. General ratings of the same order, 2, 4, 4, and 7, were also encountered in 20 animals dying of non-tuberculous complications.

TABLE 5

Groups	ANIMALS KILLED TO TERMINATE THE EXPERIMENT					
	Number of survivors	*Rating*				
		Lungs	*Liver*	*Spleen*	*Nodes*	*General rating*
Controls	0	–	–	–	–	–
J. H. 6	1	0	0	0	0	0
R_1	1	0	0	0	0	0
J. H. 16	0	–	–	–	–	–
H_{37}	2	2	1	2	2	7
		1	1	1	1	4
H_4	2	0	1	1	2	4
		1	0	0	1	2

Discussion

A controlled uniform technic has been employed to force dissociation in five different strains of tubercle bacilli. The avirulent dissociates thus produced have been stabilized by continued cultivation upon an appropriate medium for a period of four years so that there is no variation in character of growth or colony form. Virulence for guinea-pigs is likewise stable, and none of the dissociates produces more than a transitory inflammatory reaction localized at the site of inoculation.

A series of three subcutaneous injections with each of these avirulent dissociates demonstrated that all of them would protect against subsequent inoculation with the virulent dissociate of one of the strains. Here is proof that in the case of the tubercle bacillus,

the avirulent dissociate is not devoid of protective antigenic properties. It would be difficult to compare the corresponding effects of the living virulent dissociates as they would themselves cause progressive disease. It remains to demonstrate whether under identical conditions, the heat-killed dissociates from these strains are effective as vaccines.

The observations also indicate that the immunity is not type specific. The group of animals vaccinated with the homologous avirulent dissociate of the virulent one used in the test inoculation did no better than those treated with one of the other vaccines (H_4). Whether the somewhat poorer results with the other three avirulent strains are of real significance is not apparent.

Whether the differences in length of life and severity of disease in the five vaccinated groups are significant is not entirely clear. In every respect, animals given vaccines prepared from strains H_{37} and H_4 seem to have done better than the members of the other three groups.

This evidence, like that in most experiments of the kind, indicates that a limited number of animals in any mixed stock possess unusually high degrees of native resistance to the tubercle bacillus. When this is artificially reinforced by vaccination, such animals not only survive virulent re-infection, but anatomical evidences of their disease almost completely disappear. In this category belong the 6 survivors that were killed and the 20 other animals that died of causes other than tuberculosis. They constitute about one-fifth of the group as a whole. Most guinea-pigs, however, are more susceptible and in them vaccination results largely in prolonging their survival after re-infection with virulent organisms. Their lesions are naturally of a more chronic character, but the extent of their disease is frequently as great as that in unvaccinated animals.

Conclusions

Unlike many other bacteria, the avirulent dissociates of tubercle bacilli are capable of inducing an appreciable degree of immunity against re-infection with virulent strains of this organism.

Possibly the avirulent dissociates of different strains vary in their capacity to form protective antibodies. The differences demonstrated in these experiments were not sufficiently marked to warrant definite conclusions.

No evidence was produced to indicate that the immunity conferred by an avirulent dissociate is type specific for its homologous virulent dissociate.

Reference

1 Steenken, W., Jr.: Spontaneous lysis of tubercle bacilli on artificial culture media. II. Am. Rev. Tuberc., 1938, *38*, 777.