chicken embryo antigens are shown in Table I to be between 1:100 and 1:5120 by HA test and between 1:2 and 1:16 by CF test; extracted mouse brain antigens have generally yielded titers between five and ten times higher than these values. Although the CF and HA titers of suckling mouse brain S-A antigens are higher than those of the chicken embryo antigens, the mass of infected tissue in the chicken embryo frequently compensates for the lower titer per gram of tissue.

In order to ascertain the usefulness of the sucrose–acetone extracted avian antigens for the serodiagnosis of human disease, three WEE and three EEE chicken embryo antigens were compared with corresponding sucrose–acetone extracted mouse brain antigens by hemagglutination-inhibition (HI) and CF tests using a small number of patients' sera. Preliminary HI studies indicated that the chicken embryo antigens were at least as sensitive to human antihemagglutinin as the mouse brain antigens. Most of the sera reacted in the HI tests at higher dilutions using the avian antigens. In CF tests, however, the type of antigen (mouse brain or avian) giving the highest titer varied from serum to serum.

Summary. A procedure is outlined for the preparation of WEE and EEE chicken embryo CF and HA antigens. High-titered, economical antigens were prepared using the sucrose–acetone method. Differences in titer and volume between extracted chicken embryo and mouse brain antigens are discussed, and preliminary comparisons are made of the relative reactivity of human patients' sera when tested by CF and HA methods using antigens from these two sources.

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Cell Walls from *Mycobacterium tuberculosis* (BCG) as Vaccine Against *Mycobacterium leprae* Infections in Mice (32729)

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BCG vaccine, a living antituberculosis vaccine prepared from an attenuated strain of bovine tubercle bacili, provides protection against foot pad infection of mice with *Mycobacterium leprae* (1). Although ordinary preparations of BCG cell walls are not effective as vaccines against tubercle bacilli, oil-treated cell walls provide good protection against pulmonary (aerogenic) infections of mice with *Mycobacterium tuberculosis* (2). To be effective the cell walls must be combined in a dry state with a suitable oil (3). We report here a study of the enhancement of resistance against *M. leprae* infections of mice stimulated by oil-treated BCG cell walls. Of necessity the study concerned the effect of route of administration and challenge.

Materials and Methods. One cell wall vaccine was prepared in the Rocky Mountain Laboratory by methods described elsewhere

(2) from cultures of BCG originating from the Pasteur Institute. In brief, pellicle growth was disrupted in a refrigerated pressure cell, and the effluent centrifuged to sediment the cell walls, which were then resuspended and washed in distilled water by further centrifugation. After lyophilization 100 mg dry cell walls were mixed with 0.48 ml of 7-*n*-hexyloctadecane (3) and suspended in 40 ml of physiological saline containing 0.2% Tween 80 and heated at 65°C for 30 min. From the same batch of dried cell walls another lot was prepared similarly but without addition of 7-*n*-hexyloctadecane. The products were shipped under refrigeration to the National Communicable Disease Center (NCDC) where mice were vaccinated and challenged. Another batch of BCG cell walls prepared by similar methods was kindly sent in the lyophilized state by Dr. Donald W. Smith at the University of Wisconsin. Viable BCG vaccines were prepared at NCDC from a BCG strain originating from a vaccine received from Dr. S. R. Rosenthal in 1955. It was grown in Tween–albumin medium, washed and diluted in buffered saline containing 0.05% Tween 80, and standardized on the basis of turbidity measurements as described previously (4). The doses shown in Table I are dry weights.

The mice were of the CFW strain raised at the NCDC, and they were about 6 weeks old when vaccinated. The groups shown in Table I contained 20 mice, and each vaccinated group was followed by a control group of the same size. The first control group received the diluent for cell walls intravenously, the third received diluent for BCG intravenously, the sixth diluent for cell walls intradermally, and the eighth diluent for BCG intradermally. The other control groups did not receive injections at the time of vaccination. Intravenous vaccines were administered in 0.2 ml in a tail vein, and intradermal vaccines in 0.01 ml in the flank (1). An exception was made in the case of 50 μg cell walls intradermally which was given as two 0.01 ml injections also in the flank; injection of BCG vaccine in multiple sites had been found to give no more protection than that expected for the same amount of vaccine in one site

TABLE I. Vaccine Protection Against *M. leprae* Afforded by BCG Cell Walls and Intact (Living) BCG.

Vaccine			Vaccine protection[c]	
Type[a]	Amount (μg)	Route[b]	6 months	9 months
Cell wall, RML	300	iv	0.83[d]	1.17[e]
	50	iv	0.32	0.81
BCG	300	iv	1.11[e]	1.50[f]
	50	iv	1.51[d]	0.95[f]
Cell wall, UWi	50	iv	0.25	0.65[e]
Cell wall, RML	50	id	1.32	0.51[d]
	5	id	0.67	0.42[e]
BCG	50	id	1.21	2.08[e]
	5	id	1.00	1.28
Cell wall, not oil-treated	50	id	−0.03	0.08
Average AFB[g]/mouse in controls[h]			6.114	6.231

[a] Cell walls oil-treated unless otherwise stated. RML = prepared at Rocky Mountain Laboratory; UWi = prepared at University of Wisconsin.

[b] iv = intravenous; id = intradermal.

[c] Vaccine protection = $\log_{10}$ (average AFB/mouse in controls) minus $\log_{10}$ (AFB/mouse in vaccinated group). Vaccinated group contained 6–8 mice/pool except where indicated.

[d] Pool of 5 mice.

[e] Pool of 4 mice.

[f] Pool of 3 mice.

[g] AFB = acid-fast bacteria.

[h] A control group followed every vaccinated group. The average shown is the logarithm of the arithmetic mean AFB/mouse in all the control groups.

(1). After 34 days the mice were challenged by injection into the foot pad of 5×10^3 *M. leprae*, 12% of which stained solidly (5). Counts of acid-fast bacteria (AFB) in a pool of foot pad tissues harvested from 4 mice from 4 different control groups were carried out at approximately monthly intervals until the count of AFB rose above 1×10^6. (Shortly after passing this level the bacterial population levels off at its normal plateau level.) Counts were then carried out on pools of all the mouse groups. A total of 8 mice/pool had been desired; with this number differences in vaccine protection of more than

0.30 appear to be significant at usual bacterial populations (1). However, nonspecific mortality was too great to allow this. For use in estimating the protection, the numbers of AFB harvested in all the control groups were averaged arithmetically with weighting for the number of mice per group. There was no significant difference between control mice receiving diluents only and those not receiving injections at the time of vaccination.

Observations for reactions were made in the flank and in the regional (flank) lymph node were made in all the intradermally vaccinated groups, as well as in the intravenously vaccinated groups and certain control groups. For this purpose measurements with calipers were made on the mice in the first 2 cages (originally 10 mice) of most groups. The flank lymph node may be visualized in well-shaven mice if the skin is wetted with alcohol. Also flank lymph node, spleen, and lung were taken for histological studies from 4 mice (if available) from most pools counted. Tissues were fixed in neutral formalin and stained by hematoxylin, azure, and eosin, and by acid-fast.

Results. Amount of protection. The growth curve of *M. leprae* in the control mice was as follows: $<1.7 \times 10^4$ at 28 days, $<1.5 \times 10^4$ at 56, 1.1×10^5 at 88, 4.4×10^5 at 116, 6.8×10^5 at 147, 1.9×10^6 at 175 days. Counts of AFB were then carried out in all the groups and again 3 months later with the results shown in Table I.

The oil-treated cell walls provided distinct protection. When given intravenously the protection they afforded was somewhat less than that provided by an equal amount of BCG. When given intradernally they stimulated about the same protection as BCG when measured at 6 months; when measured at 9 months their protection had decreased but was still distinct. The cell walls prepared in the two laboratories were about equally effective. Cell walls that had not been treated with oil gave no protection.

Although the number of mice per pool was not as great as desired in most groups, the internal consistency of the results makes them appear reliable. Thus there is regression in protection with dilution of vaccine in all cases but one, and in that instance the harvest was so low that the number of AFB counted was very small. Also the preparations of cell walls from two different laboratories gave similar results. In addition the cell walls without oil were without effect in both harvests. The degree of protection changed between harvests, but did so consistently according to preparation and route. Differences between early and late protection have been observed previously (4).

Reactions to vaccines. At the site of intradermal vaccination the amount of induration was somewhat greater with BCG than with cell wall vaccine (average 2.45 mm vs 2.08 mm in diameter at 14 days). The flank lymph nodes were distinctly enlarged only in the mice receiving the larger dose of BCG vaccine intradermally; here they averaged 5.2 mm in diameter at 28 days as compared to averages of 2.8 mm in all other groups. The enlargement in the high dose BCG mice persisted, and the nodes still averaged 4.15 mm at 143 days. Later the enlargement decreased, but was still measurable at the harvests at 6 months and 9 months when the lymph nodes were dissected out for histology.

The histological examinations revealed nothing of note except in the lungs of mice receiving intravenous vaccines. In the mice receiving the oil-treated cell wall vaccines intravenously the lungs contained characteristic, parenchymally located nodules several hundred micra in diameter with a peripheral zone mainly of macrophages and usually a well-demarcated pale central zone of foamy structure with a few pale nuclei suggestive of macrophages. There were about 30 nodules in a section of a lobe in mice that had received 300 μg cell walls. The nodules were still present at the 9-month harvest, i.e., 10 months after vaccination. In mice receiving the lower dosage of cell walls the nodules were much less frequent and were seen in about half of the sections. In mice receiving BCG intravenously there were nodules of a similar size but they did not have the pale central areas. The chief cells were macrophages, and AFB were frequent among them. Often there were also more diffuse areas where alveoli were at least partially filled with cells, principally macrophages, sometimes with numerous AFB. These

changes were much less extensive in the mice receiving lower doses of BCG. The lungs from the 9-month harvest were only moderately improved. The histologic changes in the mice given BCG intravenously were essentially the same as those described by Youmans and Youmans (6) in mice studied much earlier after injection of BCG or H37Ra, except that in our mice the focal changes appear to have been relatively more prominent, and the diffuse macrophage reaction relatively less prominent. The microscopic proliferative changes we observed here were, of course, distinctly different from the macroscopic necrotic lesions seen in the lungs of mice following aerogenic infection of mice with virulent tubercle bacilli.

Discussion. The amount of protection against *M. leprae* provided by oil-treated BCG cell walls was about the same as that provided by intact living BCG. The intradermal route appeared to be more favorable for cell walls than for intact BCG. These comparisons are on a comparative mass basis, however. The cell walls produced much less reaction in the regional lymph node, and it may be more valuable eventually to compare the two types of vaccine on a comparative toxicity basis, since it may be more feasible to increase the amount of protection afforded by cell walls by increasing the dosage or by repeating the injection.

Previous experiments with BCG vaccination against *M. leprae* (1) had shown the intravenous route to be the most favorable, followed by the intradermal, intraperitoneal, and subcutaneous routes, in order of merit. Local (foot pad) vaccination with very small doses sometimes produced very good immunization, but the effect was irregular. In the present experiment BCG was about as good intradermally as intravenously. The cell wall vaccine appeared to be somewhat more effective intradermally than intravenously. The protection provided against *M. tuberculosis* by intradermal cell wall vaccines has not been reported; most of those studies have been carried out with intravenous vaccination and pulmonary (aerogenic) challenge. However, with those routes of vaccination and challenge it is difficult to dissociate the effect of local from systemic immunity, because much of the intravenously administered vaccine is deposited in the lungs. The pulmonary changes produced by intravenous vaccine were found in the present experiment to last at least 10 months. It would appear worthwhile to explore the intradermal route in *M. tuberculosis* experiments also, especially since this is a more acceptable route in vaccination of humans.

Cell walls that had not been treated with oil provided no vaccine protection against *M. leprae,* in parallel with the results with *M. tuberculosis* when the mice are challenged aerogenically. This is in contrast to the result when mice are challenged intravenously with a massive dose of *M. tuberculosis* 30 days after vaccination, where cell walls that have not been treated with oil also provided protection (7). The protection provided against this kind of intravenous challenge appears to be a mixture of specific and non specific effects (7).

The immunity stimulated by the oil-treated cell walls and intact (living) BCG against *M. leprae* appears to have lasted at least 6 months. When the tests are carried out as they were here, the protection observed in the first harvest may actually have been exerted earlier at the time of challenge (one month after vaccination), since activity exerted against the challenge inoculum itself can be manifested as a delay in the multiplication of *M. leprae* up to the plateau level (4). However, protection that persists until the second harvest 3 months later appears to be more lasting immunity, since it has acted by imposing a lower plateau level on the new growth of *M. leprae* 6 months or more after vaccination (4). Oil-treated BCG cell walls and intact BCG also exert a protective effect against *M. tuberculosis* in mice for at least 6 months (8).

Summary. Oil-treated cell walls of BCG were effective as vaccines against *M. leprae* infections in mice. The amount of protection was about as great as that provided by intact (living) BCG, and was distinct when given either intradermally or intravenously. In contrast cell walls that had not been treated with oil provided no protection at all, in parallel with results reported for *M. tuberculosis* by aerogenic challenge. The amount of

lymph node enlargement that followed intradermal vaccination was distinctly less with cell wall vaccines than with BCG. Characteristic long-lasting histologic foci were produced by cell walls and by BCG when administered intravenously.

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Estimation of Plasma Androgenic and Progestational Steroids in the Laying Hen (32730)

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Precise quantification of sex steroids in the chicken has been accomplished only for estrogens. Estrogens have been demonstrated in ovarian extracts (1), urine (2), and droppings (3) of the laying hen. Measurements of progesterone have been less convincing. Fraps *et al.* (4), reported the qualitative presence of progestational activity in the peripheral blood of the hen by bioassay. Layne *et al.* (5), using paper chromatography techniques, failed to find progesterone in chicken blood although they did identify this steroid in extracts of ovarian tissue. Lytle and Lorenz (6) have reported the presence of a material in ovarian venous blood that had chromatographic mobility similar to a progesterone standard. Metabolites of progesterone have not been measured. Androgens are also thought to be secreted by the hen ovary (7), but have not been quantified.

We have recently been engaged in studies to elucidate the mechanism of the induction of synthesis of a specific oviduct protein, avidin, in estrogen treated chicks by progestational and some androgenic steroids (8). Therefore, it became pertinent to attempt preliminary identification of these steroids in plasma of the mature hen using the methods presently available in our laboratory.

Materials and Methods. Nine white leghorn laying hens were anesthetized with nembutal, heparinized, and retrograde catheterization of the vena cava was accomplished by entering the left femoral vein and placing the tip of the catheter at the portion of the vena cava which drains the left ovary. This method of sampling would necessarily allow the ovarian venous effluent to be diluted by prehepatic venous blood. Plasma samples from all hens were pooled (240 ml). No attempt was made to segregate the hens according to clutch length or ovulation time.

Testosterone and Δ^4-androstenedione levels were estimated on 40 ml of the plasma pool using a double isotope technique which has been used successfully in man (9). The blank of the method determined on steroid-free human plasma is 0.010 μg/100 ml for testosterone and 0.0025 μg/ml for androstenedione. These blanks have been subtracted from the reported values.

A separate 40 ml sample of plasma was analyzed for testosterone using electron-capture gas liquid chromatography. In this meth-