Immunization of Mice by Combinations of Inactive Fractions of *Mycobacterium bovis* Strain BCG (33642)

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(Introduced by J. J. Munoz)

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Despite decades of intensive effort to obtain an effective nonviable vaccine against tuberculosis (1), the search for a substitute for presently employed vaccines containing viable attenuated mycobacteria continues. Recent work from this laboratory showed that oil-treated BCG cell walls, when injected intravenously, protect mice against aerosol challenge with *Mycobacterium tuberculosis* H37Rv about as well as does viable BCG given by the same route (2–4). The BCG cell walls also sensitize rabbits and guinea pigs to PPD (5, 6) and induce granulomatous changes in the lungs and other organs of mice (7). Whether resistance and other host responses are induced by the same or different chemical entities in the complex cell wall is not yet known. In our attempts to clarify this issue, we obtained fractions of BCG by several procedures. Several of these fractions, although inactive or nearly inactive when tested individually, confer highly significant protection to mice when combined.

Materials and Methods. Cell walls were prepared by differential centrifugation from the following bacteria disrupted in a Sorvall cell fractionator: BCG (Pasteur Institute strain 1173P2) and *M. smegmatis* (obtained from Dr. R. Bönicke, Borstel, Germany) cultivated on Sauton's liquid medium; BCG (Glaxo strain) obtained from Lederle Laboratories, Pearl River, New York; *Corynebacterium parvum* (American Type Culture Collection strain 11289) grown in thioglycollate broth; *Nocardia asteroides* (obtained from Dr. R. Bönicke) harvested from nutrient agar; and *Escherichia coli* 0113 (obtained from Dr. A. I. Braude) cultivated in an "ammonium" medium supplemented with 0.5% glucose (8). Sheep erythrocyte stroma were prepared by the method of Dodge *et al.* (9).

Chloroform extracts and waxes B, C, and D were obtained from BCG, stored at −20° prior to extraction, essentially by the methods of Aebi *et al.* (10), except that, in our laboratory, the chloroform phases were routinely passed through 0.5-μ pore size Millipore Solvinert filters. A methyl alcohol extract of acetone-washed BCG cells was prepared by refluxing the cells with methyl alcohol in a Soxhlet extractor for 48 hr (11). The BCG cells were also extracted with 5 vol of dimethyl sulfoxide by constant stirring at 37° for 16 hr. After centrifugation at 1600*g* and 20° for 0.5 hr, the supernatant fluid was then dialyzed against distilled water. The dialysate was centrifuged at 12,100*g* and 2° for 0.5 hr; lyophilization of the supernatant fluid yielded the product designated "DMSO extract." A lipid fraction was prepared from loblolly pine pollen by overnight extraction at room temperature with chloroform:methyl alcohol (2:1) (12).

One hundred mg of BCG cell walls previously extracted with ether, ethyl alcohol, and chloroform were further treated with 10 mg of lipase from *Rhizopus delemar* (generously supplied by Dr. T. Yamamoto, Osaka City University) for 3 hr at 37° in 0.1 *M* phosphate buffer (pH 6.6), washed 3 times in distilled water, and lyophilized. Other cell-wall preparations, with or without prior treatment with organic solvents, were treated

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with 0.1 *N* NaOH in 95% ethyl alcohol for 1 hr at 30° or with 0.5% KOH in absolute ethyl alcohol for 1 hr at 37°, washed 2 or 3 times in distilled water, and lyophilized. (Endotoxin is hydrolyzed by alkali more completely in an alcoholic than in an aqueous medium (M. Niwa, unpublished data).)

Some fractions of BCG were analyzed for phosphorus, total carbohydrate, and total fatty acid (amide- and ester-bound fatty acids) by methods described previously (13). The complexity of the chloroform extract was determined by centrifugal chromatography (14). The extract was dissolved in chloroform:methyl alcohol (8:2) and centrifuged onto a microparticulate bed prepared with precipitated silica and petroleum ether:diethyl ether (9:1). After centrifugation the bands were made visible with a sulfuric acid–sodium dichromate spray.

Vaccines were routinely prepared by blending in a Teflon grinder 4 drops (approximately 0.12 ml) of Drakeol 6VR (Pennsylvania Refining Company, Butler, Pa.) with 25 mg of a dry mycobacterial fraction and suspending the resulting paste in 0.2% Tween 80 in saline to the appropriate concentration. Vaccines containing 2 fractions were prepared by adding the soluble fraction dissolved in a small volume of anhydrous ether to the insoluble fraction in a dry form and stirring the mixture under a stream of air until dry. This dry combination was then treated with oil and suspended in Tween–saline as described above. The viable BCG vaccine was generously supplied by Dr. Sol Roy Rosenthal, Research Foundation, University of Illinois, Chicago, Ill., and was diluted to contain 1.5 mg of wet weight/ml (6).

Protective potency of the vaccines was assayed in mice by the test described previously (6). Briefly, 3-week-old, female, Rocky Mountain Laboratory mice were inoculated intravenously with 0.2 ml of the vaccine, and 1 month later, each mouse was infected in a Middlebrook apparatus with approximately 30–50 viable units of *M. tuberculosis* H37Rv by the pulmonary route. One month after challenge, the animals were killed with chloroform vapor and the lungs were removed for enumeration of grossly visible tubercles and for bacterial counts by culture on Dubos Tween–albumin medium. The counts of virulent organisms in the lungs of the mice in the various experimental groups were analyzed by the Fisher exact probability test as described previously (4).

Results. In our search for the BCG immunogen, we separated the ether-soluble portion of the chloroform extract into 2 fractions on the basis of solubility by the addition of 2 vol of methyl alcohol: wax B in the supernatant fluid, and a mixture of waxes C and D in the precipitate (10). Separation of waxes C and D was not attempted because of the small size of our sample. Results representative of several experiments are presented in Table I. One month after challenge mice vaccinated with 150 μg of oil-treated, unextracted cell walls (prepn. 1) had no grossly visible tubercles on their lungs and a median count of only 2.3×10^3 virulent organisms/lung. However, 6 of 20 mice vaccinated with the same quantity of cell-wall residue after extraction with solvents (prepn. 3) had detectable tubercles, and the median count for the entire group was not significantly different from that recorded for the unvaccinated controls (prep. 11). Despite the reduction in potency of the extracted cell walls, activity was not recovered in the ether-soluble portion of the chloroform extract nor in its subfractions (prepns. 4, 7, and 8). Some activity was recovered in the ether-insoluble portion of the chloroform extract (prepn. 6), but this fraction, representing only 5.9% of the original chloroform extract, contained only a small percentage of the activity of the unextracted cell walls. Cell-wall debris in the ether-insoluble portion was detected by electron microscopy.

Protection afforded by the combination of 2 inactive fractions, 150 μg each of the cell-wall residue after extraction and the ether-soluble portion of the chloroform extract (prepn. 9, Table I), was greater than that provided by any of the other fractions and equaled that conferred by vaccination with viable BCG (prepn. 10). Data from this type

TABLE I. Protection of Mice against Aerosol Challenge with *Mycobacterium tuberculosis* H37Rv by Intravenous Inoculation of Fractions of BCG (Pasteur Strain 1173 P2).

Expt.	Prepn.	Dose (μg)	Results 1 month after challenge: No. with lung lesions	Median count/lung[a]	p[b]
A	1. BCG cell walls	150	0/20	2.3×10^3	<0.002
	2. Normal controls	—	20/20	6.0×10^6	—
B	3. BCG cell wall residue after extraction with ether–ethyl alcohol and chloroform	300	0/20	7.7×10^4	<0.002
		150	6/20	2.1×10^6	>0.05
	4. Chloroform extract of intact BCG cells	300	20/20	5.6×10^6	>0.05
	5. Ether-soluble portion of prepn. 4	300	20/20	5.3×10^6	>0.05
	6. Ether-insoluble portion of prepn. 4	300	6/20	3.1×10^5	<0.003
		150	14/20	2.0×10^6	<0.003
	7. Wax B from prepn. 5	300	20/20	9.5×10^6	>0.05
	8. Waxes C and D from prepn. 5	300	20/20	7.2×10^6	>0.05
	9. Prepn. 3 combined with prepn. 5	150 + 150	1/20	1.1×10^4	<0.002
	10. Viable BCG (1.1×10^7 viable units/dose)	300 (moist wt.)	1/20	2.0×10^4	<0.002
	11. Normal controls	—	30/30	6.1×10^6	—

[a] Median count for 10 lungs.

[b] Comparison of bacterial counts in lungs of vaccinated and control mice.

of experiment suggested that 2 or more components of the tubercle bacillus were required to stimulate resistance in mice in our test system.

The complexity of the chloroform extract of the Pasteur strain of BCG was demonstrated by centrifugal chromatography. By this technique at least 4 and possibly 5 bands were resolved on the silica gel column (Fig. 1).

The BCG cell walls were treated with other reagents to determine whether the immunogen could be removed or destroyed by means other than solvent extraction; solvent extraction in our hands has not always significantly reduced the protective potency of BCG cell walls, although chloroform extracts of the Pasteur strain of BCG have consistently enhanced the potency of cell walls inactivated by solvent extraction. So far, two procedures, treatment with a lipase from *R. delemar* and with 0.1 *N* NaOH in ethyl alcohol, consistently decreased the ability of BCG cell walls to enhance resistance in mice to airborne infection (Table II). In this and other experiments, the fatty acid content of cell walls was generally reduced to a greater extent than were other components by NaOH and enzyme treatment, but whether or not the lowered fatty acid content was causally related to lowered potency has not yet been established.

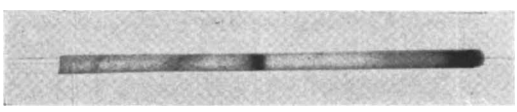

FIG. 1. Chromatogram of chloroform extract of the Pasteur Institute strain of BCG centrifuged on a column of silica in petroleum ether:diethyl ether (9:1) and made visible by spraying with sulfuric acid–sodium dichromate. The components of the sample moved from left to right in the column during centrifugation.

Data in Table I demonstrated that the ether-soluble portion of the chloroform extract of BCG enhanced the protective ability of an inactive cell wall preparation. Experiments were then designed to determine, if possible, which component of the extract was responsible for the enhancement and also whether a methyl alcohol extract of BCG

TABLE II. Protection of Mice against Aerosol Challenge with *M. tuberculosis* H37Rv by Intravenous Inoculation of BCG (Pasteur strain) Cell Walls Treated with a Lipase or NaOH.

Expt.	Cell walls treated with	Phosphorus (%)	Total carbohydrate (%)	Total fatty acid (%)	Dose (μg)	Results 1 month after challenge: No. with lung lesions	Median count/lung[a]	p[b]
1.	Ether–ethyl alcohol, chloroform	0.19	34.8	11.0	150	0/19	3.5×10^2	—
2.	Ether–ethyl alcohol, chloroform, lipase (*R. delemar*)	0.16	35.5	9.7	150	11/20	6.3×10^5	<0.003
3.	Normal controls	—	—	—	—	30/30	1.2×10^6	<0.002
1.	Not treated	0.16	24.6	17.7	150	1/20	6.7×10^3	—
2.	0.1 *N* NaOH	<0.1	ND[c]	9.6	150	13/20	2.5×10^6	0.012
3.	Normal controls	—	—	—	—	30/30	7.5×10^6	<0.002

[a] Median count for 10 lungs.

[b] Comparison of bacterial counts in lungs of groups 2 or 3 with counts in lungs of mice in group 1 in each experiment.

[c] Not done.

would similarly enhance cell wall immunogenicity (Table III). In Expt. A, wax D (generously supplied by Dr. E. Lederer) from a human strain and, in Expt. B, waxes B, C, and D which we prepared from the Glaxo strain of BCG were studied.

Wax D from Dr. Lederer failed to enhance significantly the activity of KOH-treated cell walls (prepn. 3), although in an earlier experiment these cell walls were protective when combined with the chloroform extract of the Pasteur strain of BCG. However, a combination of wax D, but not wax B or C, from the Glaxo strain of BCG and "inactive" cell walls (prepns. 11, 9, and 10) was highly effective in Expt. B. Unfortunately, the size of our wax D sample was too small to permit us to test it alone, but it is presumed that wax D alone would be inactive since the original chloroform extract was protective only when combined with cell walls.

The median count of virulent mycobacteria in the lungs of mice vaccinated with the combination of the methyl alcohol extract and the NaOH-treated cell walls (prepn. 15) also was significantly lower than the count for the control lungs (prep. 16). Neither of the components (prepns. 13 and 14) when tested individually afforded significant protection.

The above results supported the hypothesis, based on the data in Table I, that at least 2 components were required for an effective vaccine, but the data did not indicate whether both components from BCG were specifically required or whether a heterologous component could be substituted for one of the fractions. The results from several experiments in which combinations of preparations from BCG and from other sources were assayed for protective potency are presented in Table IV. Combinations of mycobacterial residues, both from BCG and from *M. smegmatis*, and chloroform extract (prepn. 3, Expt. A; prepn. 7, Expt. B; prepn. 13, Expt. C) were again found to be significantly more potent than the individual components. The p value for comparisons of counts for preparations 1 and 3, 5 (150 μg dose) and 7, and 12 and 13 was 0.012 in all cases. However, in no instance were counts of lungs of mice vaccinated with combinations of a BCG and a heterologous fraction (*C. parvum* cell walls, *N. asteroides* cell walls, *E. coli* cell walls, sheep red blood cell stroma, or pine pollen lipid) significantly lower than the count for control mice.

Discussion. The finding that at least two distinct mycobacterial components, one soluble in chloroform or in methyl alcohol and

TABLE III. Protection of Mice against Aerosol Challenge with *M. tuberculosis* H37Rv by Intravenous Inoculation of Combinations of Fractions of BCG.

Expt.	Prepn.	Dose (μg)	Results 1 month after challenge: No. with lung lesions	Median count/lung[a]	p[b]
A	1. BCG cell walls treated with KOH	150	16/20	3.7×10^6	>0.05
	2. Wax D from *M. tuberculosis* (Lederer)	300	20/20	5.5×10^6	>0.05
	3. Prepn. 1 + prepn. 2	150 + 150	14/20	1.1×10^6	>0.05
	4. Normal controls	—	30/30	6.3×10^6	—
B	5. BCG cell walls solvent- and NaOH-treated	150	19/20	2.2×10^6	>0.05
	6. Ether-soluble portion of $CHCl_3$ extract of BCG[c]	300	20/20	4.6×10^6	>0.05
	7. Wax B from prepn. 6	300	20/20	5.4×10^6	>0.05
	8. Wax C from prepn. 6	300	20/20	6.8×10^6	>0.05
	9. Prepn. 5 + prepn. 7	150 + 150	14/20	1.5×10^6	>0.05
		75 + 75	16/20	1.5×10^6	>0.05
	10. Prepn. 5 + prepn. 8	150 + 150	20/20	3.1×10^6	>0.05
	11. Prepn. 5 + Wax D from prepn. 6	150 + 150	5/20	3.0×10^2	0.012
		75 + 75	9/20	1.0×10^5	0.012
	12. Normal controls	—	30/30	2.0×10^6	—
C	13. BCG cell walls solvent- and NaOH-treated	300	8/20	2.4×10^6	>0.05
	14. Methyl alcohol extract of BCG	300	20/20	1.5×10^6	>0.05
	15. Prepn. 13 + prepn. 14	150 + 150	2/20	1.7×10^4	<0.002
	16. Normal controls	—	25/25	2.4×10^6	—

[a] Median count for 10 lungs.

[b] Comparison of bacterial counts in lungs of vaccinated and control mice.

[c] Glaxo strain.

the other presumably chloroform-insoluble and associated with the cell-wall framework, are apparently required to stimulate resistance in mice to airborne infection with *M. tuberculosis* H37Rv may explain why investigators who attempted to isolate from mycobacteria an effective immunogen as a single chemical entity failed. In our experience (not reported here) crude chloroform extracts sometimes were effective vaccines, but when the bulk of the cell-wall debris was removed, potency also disappeared. Hoyt *et al.* (15) also found that a combination of wax and mycobacteria lost its immunizing power when most of the bacteria were removed by ultracentrifugation.

Our first explanation for these results was that potency of crude extracts was due to contaminating cell walls; but, when protective potency was achieved by combination of 2 inactive fractions, alternative hypotheses were sought. Others have reported that combinations of mycobacterial fractions have induced or intensified several host responses. Williams and Dubos (11) found that two of their subfractions of a methyl alcohol extract of mycobacteria acted synergistically to protect mice challenged intravenously with *M. bovis*. Smith and Kubica (16) reported that an ethyl alcohol extract of mycobacteria mixed with a defatted bacillary residue significantly increased the resistance of guinea pigs to subcutaneous challenge with *M. tuberculosis*. Finally, Raffel (17) discovered that guinea pigs developed delayed hypersensitivity to tuberculoprotein only when purified mycobacterial wax was administered with the protein. Since mycobacteria are well known

for their adjuvant properties, it is conceivable that one component in our vaccine, the wax D fraction of the chloroform extract, serves primarily as an adjuvant to "stimulate" the host to respond in a significant manner to the immunogen present in the second fraction of the vaccine. A similar suggestion was offered by Williams and Dubos (11) to explain their findings.

Several other hypotheses to explain our

TABLE IV. Protection of Mice against Aerosol Challenge with *M. tuberculosis* H37Rv by Intravenous Inoculation of Combinations of Heterologous and BCG Fractions.

Expt.		Prepn.	Dose (μg)	Results 1 month after challenge: No. with lung lesions	Results 1 month after challenge: Median count/lung[a]	p[b]
A	1.	BCG cell walls treated with 0.1 N NaOH	150	13/20	1.7×10^6	>0.05
	2.	Ether-soluble portion of $CHCl_3$ extract of BCG	300	20/20	9.7×10^6	0.012[c]
	3.	Prepn. 1 + prepn. 2	150 + 150	9/20	1.8×10^3	0.012
	4.	Normal controls	—	30/30	2.6×10^6	—
B	5.	DMSO extract of BCG	300	3/20	5.9×10^1	<0.002
			150	6/16	4.5×10^1	0.008
	6.	Ether-soluble portion of $CHCl_3$ extract of BCG	300	19/19	7.2×10^6	>0.05
	7.	Prepn. 5 + prepn. 6	150 + 150	1/20	1.4×10^3	<0.002
			75 + 75	0/19	6.9×10^3	<0.002
	8.	*C. parvum* cell walls	300	20/20	3.4×10^6	>0.05
	9.	Prepn. 6 + prepn. 8	150 + 150	19/19	2.2×10^6	>0.05
	10.	Normal controls	—	29/29	3.2×10^6	—
C.	11.	Ether-soluble portion of $CHCl_3$ extract of BCG	300	19/20	4.4×10^6	>0.05
	12.	*M. smegmatis* cell walls	150	15/20	1.8×10^6	>0.05
	13.	Prepn. 11 + prepn. 12	150 + 150	7/20	8.1×10^1	0.012
	14.	*N. asteroides* cell walls	150	20/20	3.6×10^6	0.012[c]
	15.	Prepn. 11 + prepn. 14	150 + 150	10/20	1.1×10^6	>0.05
	16.	Normal controls	—	30/30	1.2×10^6	—
D	17.	BCG cell wall residue after extraction with ether–ethyl alcohol and $CHCl_3$	150	16/20	4.1×10^6	>0.05
	18.	$CHCl_3$–methyl alcohol extract of loblolly pine pollen	150	20/20	1.9×10^6	>0.05
	19.	Prepn. 17 + prepn. 18	150 + 150	16/19	1.3×10^6	>0.05
	20.	Ether-soluble portion of $CHCl_3$ extract of BCG + *E. coli* 0113 cell walls	150 + 150	6/20	1.7×10^6	>0.05
	21.	Normal controls	—	30/30	2.4×10^6	—
E	22.	Ether-soluble portion of $CHCl_3$ extract of BCG	300	17/20	3.0×10^6	>0.05
	23.	Sheep red blood cell stroma	300	20/20	6.4×10^6	>0.05
	24.	Prepn. 22 + prepn. 23	150 + 150	12/20	3.8×10^6	>0.05
	25.	Normal controls	—	20/20	3.9×10^6	—

[a] Median count for 8–10 lungs.

[b] Comparison of bacterial counts in lungs of vaccinated and control mice.

[c] The count for the vaccinated mice was significantly higher than the count for the control mice.

results cannot as yet be excluded. The soluble component may impart a lipophilic character to the surface of the inactive cell walls, which may contain the essential immunogen, so that the cell walls are distributed in the oil phase of the vaccine rather than in the aqueous phase. Extracted cell walls which do not associate with the oil droplets do associate with them after combination with the chloroform-soluble extract (14). Barclay *et al.* (7) have shown that, in all effective vaccines studied, the cell walls are always associated with the oil droplets. Finally, the soluble component may be the only essential ingredient of a good vaccine, but the active substance must be suitably dispersed on the surface of a nonspecific carrier substance, such as the cell wall, to exert its full effect. Although our data are not conclusive, the failure of combinations of the chloroform extract and heterologous "carrier" substances makes this hypothesis less attractive than the others.

We do not yet know whether the wax D fraction and the methyl alcohol extract contain the same or different active components. Preliminary studies of these preparations by centrifugal chromatography procedures have revealed that both contain multiple components. Attempts are now being made to isolate and identify the active component(s) in these preparations.

At present we cannot explain why Lederer's wax D from a human strain of tubercle bacillus when combined with "inactive" cell walls failed to protect mice, while the wax D preparation from BCG prepared in our laboratory in combination with "inactive" cell walls was potent. Perhaps the differences in strain and in preparative procedures are responsible for the difference in effectiveness. Chromatograms of the two wax D products indicated a dissimilarity in their composition, with certain components being absent in one and present in the other while at the same time the components in common varied in concentration.

Summary. In an attempt to identify the mycobacterial cell wall components which enhance resistance in mice to airborne infection with *Mycobacterium tuberculosis* H37Rv, fractions of BCG were prepared by organic solvent extraction and by alkali or lipase treatment. Although certain of these fractions, when tested individually, were impotent in our protection test, vaccines containing combinations of solvent-extracted, alkali-treated, or lipase-treated cell walls and of an inactive chloroform or methyl alcohol extract were significantly effective. The wax D fraction, but not the wax B or C fractions, of the chloroform extract in combination with "inactive" cell walls was highly protective. Since combinations of either the "inactive" BCG cell wall residue or the BCG chloroform extract with heterologous substances failed to enhance resistance of mice, two or more specific and distinct mycobacterial components *may* be required in an effective vaccine.

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Zygote Transfer to Facilitate Altered Expression of Immunoglobulin Light Chain Phenotypes in Homozygous Rabbits* (33643)

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Rabbits of genotype b^4b^5, heterozygous for the light polypeptide chains of immunoglobulins, generally have approximately twice as much b4-γG than b5-γG (1, 2).[1] However, a persistent alteration in the quantitive expression of these allelic allotypes occurs in heterozygous rabbits exposed during lethal and neonatal life to antibody specific for the allotype inherited from the gene of the father (3). Thus, when b^4b^5 homozygous mothers are immunized against the paternal b5 allotype the b^4b^5 heterozygous offspring, throughout their lives, have very low serum b5 allotype and relatively high compensatory levels of the b4 allotype; e.g., one rabbit had b4 to b5 ratios of 300:1 at 5 months, 71:1 at 1 year, 63:1 at 2 years and 19:1 at 3 years (4, 5). "Allotype suppression" can be effected either by uterine transfer of antiallotype antibody or by injection of newborn heterozygous rabbits with antiallotype antisera directed against the paternal allotype (3, 5). In heterozygous suppressed animals, the total γG levels appear normal and a compensatory increased production of the allelic allotype accounts for most of the immunoglobulin in the sera (3, 5).

The question arose as to whether all immunoglobulins with light chains controlled at the *b* locus could be suppressed by injection of antiallotype antibody into homozygotes. Would this lead to ahypogammaglobulinemia or would a compensatory increase of light chain synthesis occur at another locus in order to maintain normal levels of immunoglobulins? In fact, 10–30% of the molecules from homozygous or heterozygous rabbits lack allotypic specificities controlled by the *b* locus suggesting that some light chains are controlled by genes other than at the *b* locus (1, 2).

It was not feasible to attack the problem directly since a newborn homozygous rabbit, e.g., b^5b^5, must come from a mother having the b^5 gene and thus at birth will have a relatively large amount of the b5 allotype as a result of uterine transfer. Anti-b5 injected into this newborn would react with the b5 allotype to form antigen–antibody complexes and this may prevent the free antibody from being effective on the cell population synthesizing b5. Dubiski (6) minimized uterine transfer of the b5 allotype by using b5 suppressed b^4b^5 heterozygous mothers. He succeeded thereby in obtaining significant suppression of b5 light chains in the b5 homozygous offspring (6). However, by this precedure, half the offspring are b^5b^5 homozygotes and half are b^4b^5 heterozygotes, making it difficult to distinguish the genotype of the newborn. Furthermore, b5 allotype suppressed rabbits are still producing small

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[1] Allotypic specificities Ab4, Ab5, and Ac7 are abbreviated to b4, b5 and c7; the genes are b^4, b^5, and c^7.