Adjuvant Activity of Erythrocyte Isoantigens

Abstract. *Erythrocyte isoantigens determined by the* B *blood-group locus of chickens enhance the immune response to weak isoantigens. This adjuvant action occurs if the antigens are present on the same erythrocyte, and the recipients are capable of responding immunologically to the B antigens.*

That chicken erythrocyte isoantigens determined by the *B* blood-group locus behave as an adjuvant with regard to the immunogenicity of other less antigenic isoantigens, determined by the *A, C, D,* and *L* blood-group loci, has become apparent in our attempts to produce blood-typing antiserums (*1*). This property may extend to isoantigens of other species, including the H-2 in mice (*2*) and the Rh in man (*3*).

Most antigens determined by the *B* blood-group locus in chickens are potent immunogens in that nearly all birds produce high titers of antibodies after immunization with these antigens. However, *A* blood-group system antigens are usually weak immunogens, with few birds responding even after prolonged immunization. The purpose of our study was to determine some of the requirements of the adjuvanticity of *B* system antigens for *A* system antigens.

In the first experiment, we set out to ascertain whether the adjuvant effect occurs equally well when *A* and *B* system antigens are on the same or separate erythrocytes. Three groups of 1-month-old chickens from a partially inbred line (*4*) received weekly inoculations of erythrocytes; the first two inoculations were given intramuscularly and the remainder intravenously. Group 1 received erythrocytes having foreign A_2 antigens only; group 2 received erythrocytes with both foreign A_2 and B_3 antigens; and group 3 received a mixture of two types of erythrocytes: those having only foreign A_2 antigens and those having only foreign B_3 antigens. The numbers of inoculated erythrocytes were adjusted so that groups 2 and 3 received approximately equal quantities of each antigen. Hemagglutinin titers were determined after six inoculations.

In the second experiment the antibody response to *A* system antigens of birds rendered completely or partially tolerant of *B* system antigens was investigated. Seventeen newly hatched chicks (genotype $A^1/A^1,B^1/B^1$), from the same partially inbred line, received intravenous inoculations of a leukocyte-poor suspension of erythrocytes having foreign B_2 antigens (donor genotype $A^1/A^1,B^1/B^2$). Three further inoculations were given at 4- to 6-day intervals. Four weeks after the last tolerance-conferring injection the birds received three weekly intravenous inoculations of erythrocytes having foreign A_2 and B_2 antigens (donor genotype $A^1/A^2,B^1/B^2$). Their serums were titrated for hemagglutinins 1 week after the last inoculation. Control groups consisted of nontolerant birds, from the same hatch, which received either A_2 antigens only, or A_2 and B_2 antigens on the same erythrocyte.

Table 1. Adjuvant activity of B antigens.

Immunizing antigen(s)	Total	No. of Birds	
		Producing B3 antibodies*	Producing A2 antibodies*
A_2	12	0	1†
A_2 and B_3 (separate RBC)	11	11‡	0
A_2 and B_3 (same RBC)	11	11‡	11†

* After six weekly injections. † Titers from 1 : 4 to 1 : 32. ‡ All titers > 1 : 32.

Only one out of 12 birds produced antibodies to the A_2 antigen when immunized with this antigen alone (Table 1). The striking adjuvant activity of B_3 antigens is evident since all birds immunized with B_3 and A_2 antigens (on the same erythrocyte) produced A2 antibodies in addition to B3 antibodies. Absorption studies showed that these antibodies were distinct, since either one could be absorbed without significantly decreasing the titer of the other. That both antigens have to be present on the same carrier erythrocyte for the B antigens to exert their adjuvant action is also apparent. When the two antigens were on separate erythrocytes, all the birds exhibited a normal antibody response to the B_3 antigen but none responded to the A_2 antigen.

The adjuvant property of an antigen determined by a different *B* allele (B^2) is apparent from the results of the second experiment (Table 2, groups 1 and 2). No adjuvant effect existed when the recipient birds had been rendered completely tolerant of the B_2 antigen (group 4). Moreover, only one-half of the birds responded to the A_2 antigen when the tolerance was incomplete (group 3). Those birds which did respond had low titers of antibodies.

Our studies disclose two requirements for the expression of the adjuvant property of *B* system antigens; both antigens must be present on the same erythrocyte, and the recipients must be capable of reacting immunologically against the B antigens. The mechanism of the B antigen adjuvant effect is obscure. One possibility is that the adjuvant action occurs during macrophage participation. This is compatible with theories that emphasize the primacy of phagocytosis and processing of antigen by macrophages in antibody formation (*5*). If erythrocytes carrying foreign B antigens are more readily phagocytized than erythrocytes carrying only foreign A antigens, this alone may account for the greater antigenicity of B antigens. It is unlikely that a mechanism exists for the selective exclusion of A antigens from macrophages if they reside on the same erythrocyte that carries a foreign B antigen. However, it may be possible for chicken macrophages to discriminate between isoantigens on different erythrocytes. Preferential phagocytosis of erythrocytes by mouse macrophages, dependent on phylogenetic relationship, has been reported (*6*).

Rat macrophages may be fully capable of phagocytizing an antigen to which tolerance had been induced (*7*). If such is the case with the B-tolerant birds their failure to respond to A antigens in an enhanced fashion may indicate (i) enhanced phagocytosis of

Table 2. Adjuvant activity of B antigens in birds completely or partially tolerant of B antigens.

Recipient group	Birds (No.)	Donor erythrocyte antigens	Antibodies produced			
			B2		A2	
			No. of birds	Titer*	No. of birds	Titer*
1. Nontolerant	16	A_2	0		1	2
2. Nontolerant	18	A_2; B_2†	18	4-64(21.8)	16	1-64(7.3)
3. Partially tolerant‡	10	A_2; B_2†	10	1-32(4.3)	5	1-2(1.3)
4. Completely tolerant	7	A_2; B_2†	0		0	

* Range and geometric mean of reciprocal titer. † Both antigens present on some erythrocyte. ‡ Includes all birds which received tolerance-conferring injections and subsequently produced B antibodies.

A antigens is not the explanation of the B antigen adjuvant effect or (ii) the antigen processing machinery of macrophages from tolerant animals may be deficient not only with regard to the specific B antigens but also to the serologically distinct A antigens present on the same particle. From our study we do not know whether the birds tolerant of the B antigens responded less well to the A_2 antigens than one would expect of nontolerant birds immunized with A_2 alone, since so few of the latter responded (Table 2, group 1).

The possibility that macrophages which phagocytize two serologically distinct antigens pass on the necessary stimulus for the production of antibody to both antigens seems appealing since we have previous evidence that lymphoid cells which produce antibodies to *A* system antigens belong to the population of cells which produce antibodies to *B* system antigens (*8*). The ability to demonstrate that cells may produce two antibodies with different specificities may in part depend on the number of macrophages which phagocytize and suitably process both antigens. This may be a random phenomenon except in the situation where both antigens are present on the same carrier particle.

The adjuvant property of isoantigens may be valuable in the production of erythrocyte and leukocyte typing reagents which are ordinarily difficult to produce. By the same token it may complicate tissue typing studies in the human population since weak tissue antigens may acquire enhanced immunogenicity under the influence of a strong tissue antigen (*9*). Whether advantage can be taken of the isoantigen adjuvant phenomenon in inducing effective immunity to weak viral or tumor specific antigens has yet to be determined.

L. W. SCHIERMAN
R. A. MCBRIDE
Department of Surgery, Mount Sinai Hosptial, New York 10029

References and Notes

1. The letter designation for the various chicken blood-group systems are specific and correspond to those used by other workers whereas the numerical superscripts, which designate specific alleles, apply only to the strain of chickens used in this study. Subscripts indicate antigen, and numbers on the line indicate antibody.
2. D. B. Amos, *Ann. N.Y. Acad. Sci.* **97**, 69 (1962).
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4. Inbreeding coefficient approximately equal to 0.60. Isoimmunization and blood-group studies in the last eight generations suggest that this line is genetically homogeneous for blood group systems other than *A, B, C, D* and *L*.
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9. This suggestion was made to us by Prof. M. Simonsen.
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Human Fibroblasts Infected with Rubella Virus Produce a Growth Inhibitor

Abstract. *A protein that inhibited mitosis of normal human diploid cells was demonstrated in extracts of WI-38 cells that were infected with rubella virus and that had gone into mitotic arrest subsequent to infection. A possible mechanism for the pathogenesis of the rubella syndrome is suggested.*

Human diploid fibroblasts infected with rubella virus in vitro are unchanged morphologically. When subcultured, however, these cells show effects ranging from mitotic inhibition to chromosomal breakage (*1, 2*). Infection of human fetal cells in vivo results in a complex of congenital anomalies referred to as the rubella syndrome. Naeye and Blanc (*3*) performed histopathologic analyses of the tissues of infants with rubella syndrome and found evidence of a general retardation in growth owing to mitotic inhibition. Cell strains carrying viruses derived from fetuses infected with rubella were studied in vitro by Rawls and Melnick (*4*), who found a slowed mitotic rate.

We have studied the mechanism by which rubella virus causes mitotic inhibition of WI-38 human diploid lung cells (*5*) and have found that infection induces the formation of a specific protein that slows mitosis. We call this substance rubella virus-induced mitotic inhibitor (RVIMI). Human diploid fibroblasts (WI-38) in the 18th to 28th passage in stoppered monolayer cultures containing about 4×10^6 cells were infected either with $10^{6.3}$ plaque-forming units (PFU) of rubella virus grown and titrated in BHK-21 cells (*6*) or with $10^{5.0}$ $TCID_{50}$ (tissue culture infectious dose, 50 percent effective) of rubella virus grown and titrated in WI-38. In both cases the growth and maintenance media were Eagle's basal medium and 10 percent fetal bovine serum. One week after infection the cells were suspended by treatment with trypsin. The suspension of cells was divided in half and subcultivated to two fresh bottles. A week later, or 2 weeks after they were inoculated with virus, infected and control cultures were treated with trypsin, washed, and resuspended in phosphate-buffered saline or in the maintenance medium. By the time of harvesting, the infected cultures showed visible evidence of arrested growth, while control cultures were confluent. After the cells were counted, the volumes were adjusted so that both suspensions contained 5×10^6 cells per milliliter. The suspensions were then subjected to three cycles of freezing and thawing, followed by 7 minutes (interrupted by shaking) of sonic oscillation in a Raytheon DF101 oscillator at 1 amp. The cell lysates were centrifuged for 2½ hours at 32,000*g* in a Sorvall centrifuge, a treatment which sediments infectious rubella virus (*7*). To remove additional live virus, we exposed the supernatants to 5 minutes of ultraviolet (UV) radiation delivered by a Westing-

Table 1. Effect of cell extracts on growth of WI-38 cells. To petri dishes were added 1×10^6 to 2×10^6 WI-38 cells. Extracts were added immediately. After 2 hours, about 30 to 40 percent of the cells were attached. On day 1, dishes to which nothing had been added showed a 25 percent increase in cell count above the number attached at 2 hours. Cells counts were performed in replicate and averaged. The error of the method equals ± 200,000. The dilutions were calculated by dividing the amount of undiluted cell extract added to 4 ml total volume by 4 ml.

Dilution	Cell counts 2 days after seeding ($\times 10^5$) in experiment			
	1	2	3	4
Rubella-infected cell extract				
1/20	4.5	12.7	8.0	8.5
1/40	6.2	11.9	8.5	8.0
1/80	7.9	13.4	10.0	9.9
1/160	8.6	17.5	11.9	11.4
Control-cell extract				
1/20	9.0	18.3	10.1	9.6
1/40	9.2	19.0	12.5	12.7
1/80	8.4	18.7	11.8	11.5
1/160	8.5	18.0	12.0	12.2
No extract				
	8.5	19.3	12.2	12.4