INDUCTION OF RESISTANCE TO ANTIBODY-MEDIATED CYTOTOXICITY

H-2, Ia, and Ig Antigens are Independent Entities in the Membrane of Mouse Lymphocytes*

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One of the main problems in the genetic analysis of a complex antigenic system is cross-reactivity of the antibodies defining individual antigens. Because of cross-reactivity it is often difficult, if not impossible, to determine the relationship between genetic structure on the one hand and molecular interrelationships among the antigens on the other hand. Although, theoretically, such a relationship can be elucidated by biochemical methods, the biochemical approach is complicted by the uncertainty whether the isolated products represent pieces of the membrane, molecules, or molecular fragments. Moreover, the biochemical methods are still too complicated to be used routinely in an ordinary serological analysis.

Recently, two methods have become available that by-pass the difficulties indigenous to the biochemical analysis, and yet provide important information about the molecular interrelationship of individual antigens. Both methods are based on the assumption that independent molecules float freely in the cell membrane (1) and can be aggregated when combined with antibodies (2). Of the two methods, namely, redistribution of antigens after exposure to fluorescent antibodies ("capping", cf. ref. 2) and acquisition of specific resistance to complement-dependent lysis after antigen redistribution ("lyso-strip", cf. ref. 3, 4), only the former has been utilized in the studies of the mouse *H-2* complex (5). In this report we describe an application of the lysostrip method to the *H-2* system and provide evidence for independent movement in the lymphocyte membrane of *H-2*, Ia, and Ig antigens.

Materials and Methods

Principle of The Lysostrip Method. The method is carried out in three stages. In the first stage lymphocytes are incubated with monospecific mouse alloantiserum (e.g., anti-*H-2.2*) and the corresponding antigenic sites are coated with alloantibodies. In the second stage, a second layer of antibodies is added by incubating the cells with a xenogeneic antimouse Ig serum, and aggregation and capping of the antigenic sites are allowed to occur. In the third stage, the cells are tested in the standard cytotoxic test. If significant redistribution and capping occurred, large areas of the cell surface should now be devoid (stripped) of the particular antigens (i.e., *H-2.2*), or they should be in a

* Supported by grants AI 11650 and AI 11879 from the National Institutes of Health.

state resistant to lysis when additional antibody of the same specificity (i.e., anti-*H-2.2*) and complement are added. On the other hand, antigens present on molecules other than those carrying the redistributed antigens (e.g., *H-2.33* in the case of $H\text{-}2^b$ lymphocytes) should be distributed evenly throughout the membrane and the cells should be killed when exposed to antibodies against these antigens (i.e., anti-*H-2.33*) in the presence of complement.

Cell Suspensions. Spleen or thymus were placed into a Petri dish containing Hanks' balanced salt solution (HBSS)[1], and adhering fat tissue was removed. The organs were then fragmented with a spatula and cells released by teasing. The suspension was flashed back and forth in a Pasteur pipette, large fragments were allowed to sediment for 5 min in a test tube and the supernate was transferred into another tube and centrifuged. Erythrocytes were destroyed by washing the cells twice in amonium chloride Tris (ACT), followed by one washing in HBSS. The cells were then counted and diluted to a required concentration. The whole procedure except for the ACT treatment was carried out in the cold.

Induction of Resistance to Lysis. The method used to induce resistance to lysis (lysostrip) was that of Bernoco et al. (3) with the following modifications. Spleen cells were suspended in Hanks' solution and divided into aliquots, each containing 5×10^6 cells. The suspensions were centrifuged, the supernates removed, the cells resuspended in *H-2* alloantisera appropriately diluted in phosphate-buffered saline (PBS) (see Results) and incubated for 2 h on ice. After the incubation, the cells were washed twice in the cold with BSS and incubated with xenogeneic antimouse-Ig serum (diluted in PBS) for 1 h on ice. The cells were then washed twice in BSS, resuspended in RPMI medium containing 10% heat-inactivated normal mouse serum (NMS) (pool) and cycloheximide (50 μg/ml), to give a final concentration of 2×10^6 cells/ml, and incubated for 1 h at 37°C. The suspension was then cooled to 0°C, washed twice with BSS, and the incubation with the anti-Ig serum was repeated one more time. At the end of the second Ig-treatment, the suspension was used without washing in the cytotoxicity test which was performed at room temperature (except for the incubation period with complement).

Cytotoxic Test. Susceptibility or resistance to cytotoxicity after antisera treatment was determined by using the two-stage microcytotoxicity test of Amos and his co-workers (6), modified as described previously (7). Live and dead cells were distinguished by using an inverted phase-contrast microscope. Commericially available normal guinea pig serum (Grand Island Biological Company, Grand Island, N. Y.), diluted 1:8, was used as a source of complement.

Alloantisera. The *H-2* and Ia antisera used and the antibodies they contain are listed in Table I. The antisera were produced using an immunization regimen described previously (8, 9). The specificity of the antisera was determined by testing them against a panel of inbred strains and congenic lines representing all major *H-2* haplotypes. The anti-Thy-1.2 serum was produced using the immunization schedule of Reif and Allen (10).

Xenoantisera. The following xenoantisera against mouse Ig were tested: horse antimouse-Ig-fluorescenin conjugated (Roboz Surgical Instruments Company, Washington, D. C.), goat antimouse-gammaglobulin and goat antimouse-whole serum (Meloy, Springfield, Va.), rabbit antimouse-Ig-(RAMIG), and goat antimouse-Ig(GAMIG) (γ κ.). The latter two antisera were gifts from Dr. E. S. Vitetta, University of Texas Southwestern Medical School.

Mice. The strains of mice used and their *H-2* genotypes are listed in Table II. All mice were produced in our animal colony at the University of Texas Health Science Center.

Results

Technical aspects of the lysostrip method. The outcome of the lysostrip test is affected by many variable, some of which are discussed below:

SOURCE OF ANTI-IG SERUM. Three sources of anti-Ig sera were tested: horse, rabbit, and goat. All three types of antisera apparently contained natural antibodies against mouse lymphocytes, as they killed 100% spleen cells, and for

[1] *Abbreviations used in this paper:* As, antiserum; ACT, ammonium chloride Tris; C', complement; GAMIG, goat antimouse immunoglobulin; HBSS, Hanks' balanced salt solution; NMS, normal mouse serum; PBS, phosphate-buffered saline; RAMIG, rabbit antimouse immunoglobulin.

TABLE I

Alloantisera Used

Serial no.	Recipient	Donor	*H-2* haplotype combination	Antibodies present
K-26	(B10 × A)F_1	AQR	(*b/a*)*y1**	*H-2.17*
K-30	(B10.D2 × A)F_1	HTI	(*d/a*)*i*	*H-2.33*
K-119	(B10 × A)F_1	B10.S	(*b/a*)*s*	*H-2.19*
K-131	(B10.A × A)F_1	B10.D2	(*a/a*)*d*	*H-2.31*
K-302	(B10.D2 × A)F_1	B10.A(2R)	(*d/a*)*h2*	*H-2.2*
K-304	(A.BY × B10.AKM)F_1	B10.A	(*b/m*)*a*	*H-2.4*
K-210	B10.T(6R)	AQR	(*y2*)*y1*	Ia.1
K-212	(A.TL × B10)F_1	B10.HTT	(*t1/b*)*t3*	Ia.3.4
K-226	AKR	C3H	(*k*)*k*	Thy-1.2

* Recipient in parentheses, donor outside.

TABLE II

H-2 Genotypes of Strains Used As Spleen Cell Donors

Strain	*H-2* haplotype	Origin of *H-2* regions and subregions					
		K	*IA*	*IB*	*IC*	*S*	*D*
B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*
AQR	*y1*	*q*	*k*	*k*	*d*	*d*	*d*
B10.HTT	*t3*	*s*	*s*	*s*	*k*	*k*	*d*

this reason, they had to be absorbed by mouse thymocytes. After the absorption, the horse antiserum and some of the commercially available goat antisera proved to be completely ineffective in inducing resistance to cytotoxicity. The rabbit antisera, when absorbed with 3×10^8 thymocytes, still displayed relatively high background killing (between 25 and 35%, cf. Table V); further absorption decreased the efficiency of the antisera without significantly lowering the background cytotoxicity. The most satisfactory results were obtained with goat antimouse Ig sera, particularly with the anti-γ, κ serum provided by Dr. Vitetta. The latter antiserum was, therefore, used in most of the experiments, after absorption with 10^8 thymocytes/300 μl of antiserum. All antisera had to be heat-inactivated, otherwise a substantial loss of cells occurred during the procedure.

CONCENTRATION OF ALLO AND XENOANTIBODIES. The results of the lysostrip procedure depend to a large degree on the concentration of both the *H-2* alloantisera and the anti-Ig xenoantisera. The optimal concentration of antibodies for induction of resistance to cytoxicity varied from serum to serum and had to be determined empirically. With the *H-2* alloantisera, best results usually were obtained at antibody concentration that kills 80% of lymphocytes in the cytotoxicity test. Heat-inactivated *H-2* antisera were ineffective in inducing resistance to cytotoxicity.

In some cases, the anti-Ig antibodies might have reacted with the receptors on splenic B cells and these cells then might have been killed after the addition of complement, causing relatively high background cytotoxicity. To avoid this possibility, the cells, before incubating them with alloantisera, were treated with the anti-Ig antibodies, with the aim to cap or strip the cells of their Ig receptors. In other instances, a similar effect might have been achieved by the addition of a second or third cycle of antisera treatment (see below).

TARGET CELLS. Two types of cells were tested as targets for the induction of resistance to cytotoxicity: thymocytes and lymphocytes. Thymocytes usually displayed lower background cytotoxicity but their general use was hindered by the fact that their content of Ia antigens (7, 11), as well as some *H-2* antigens (J. Klein, unpublished data) is very low.

LENGTH OF THE INCUBATION PERIOD WITH ANTI-IG. Although one cycle of anti-Ig treatment was usually sufficient to induce significant reduction in susceptibility to cytotoxicity, background levels of susceptibility were achieved only after a second or a third cycle.

Molecular Independency of K and D Region Antigens. The private *H-2* antigens (i.e., antigens restricted to a very limited number of independent *H-2* haplotypes) can be classified into two series, K and D, with antigens in each series being mutually exclusive (12, 13). Each *H-2* haplotype of any of the well-defined inbred strain is, therefore, characterized by two private antigens, one in the K series and another in the D series. According to the two-locus model (13), the private antigens are controlled by alleles at two loci, *H-2K* in the *K* region and *H-2D* in the *D* region. It is assumed that the products of the *H-2K* and *H-2D* loci represent distinct antigenic molecules that can move independently in the cell membrane. Were this the case, one should be able, using the lysostrip method, to induce resistance to antibodies against the *H-2K* antigens and not to affect sensitivity to those against *H-2D* antigens and vice versa. This postulate was tested and confirmed in several antigen combinations. Table III shows the results of the test with the $H\text{-}2^b$ haplotype of strain B10 characterized by private antigens *H-2K.33* (detected by antiserum K-30) and *H-2D.2* (detected by

TABLE III

Susceptibility of B10 Spleen Cells to Cytotoxic Killing After Anti-H-2 and GAMIG Treatment

Pretreated with:	Tested with:	Dead cells at dilution of As:		
		1:4	1:8	1:16
		%	%	%
—	K-302	85	75	60
K-302(1:2) + GAMIG(1:4)	K-302	12	15	12
K-302(1:2) + GAMIG(1:4)	K-30	75	80	75
—	K-30	75	70	70
K-30(1:8) + GAMIG(1:4)	K-30	25	20	20
K-30(1:8) + GAMIG(1:4)	K-302	85	70	50
GAMIG(1:4)	NMS + C′	12	10	10

antiserum K-302). Pretreatment of B10 spleen cells with anti-*H-2K.33* induced resistance to anti-*H-2K.33* but not to anti-*H-2D.2*, and vice versa. In Table IV, the $H\text{-}2^d$ haplotype of strain B10.D2 was similarly tested. The haplotype is characterized by private antigens *H-2K.31* (detected by antiserum K-131) and *H-2D.4* (detected by antiserum K-304). The table shows that after the treatment of B10.D2 spleen cells with anti-*H-2K.31* antibodies, the cells are resistant to cytotoxicity in the presence of anti-*H-2K.31* and complement, but are killed by anti-*H-2D.4* and complement, and that the same effect is also observed in the reciprocal combination.

Similar results also were obtained with strains B10.HTT ($H\text{-}2^{t3}$) and AQR ($H\text{-}2^{y1}$). These are discussed in the following section. All these data support the hypothesis that the *H-2K* and *H-2D* gene products are separate antigenic entities that are not spatially interconnected in the cell membrane.

TABLE IV

Susceptibility of B10.D2 Spleen Cells To Cytotoxic Killing After Anti-H-2 and GAMIG Treatment

Pretreated with:	Tested with:	Dead cells at dilution of As:		
		1:4	1:8	1:16
		%	%	%
—	K-304	95	85	50
K-304(1:4) + GAMIG(1:4)	K-304	28	28	20
K-304(1:4) + GAMIG(1:4)	K-131	100	90	70
—	K-131	80	60	50
K-131(1:8) + GAMIG(1:4)	K-131	30	18	15
K-131(1:8) + GAMIG(1:4)	K-304	90	80	50
GAMIG(1:4)	NMS + C'	10	10	10

Molecular Independency of Ia and H-2 Antigens. Ia antigens are genetically controlled by the *I* region of the *H-2* complex and characterized by more restricted tissue distribution (e.g., the antigens are predominant on B lymphocytes and much less expressed on T cells, cf. 7, 11, 13) and by lower molecular weight (around 30,000 daltons cf. 14, 15), then classical *H-2* antigens. Genetic and biochemical data suggest that the Ia antigens are present on different molecules than the *H-2* antigens (15–18). Tables V and VI provide evidence that Ia and *H-2* gene products also behave as independent moieties in the cell membrane. The B10.HTT strain (Table V) carries the $H\text{-}2^{t3}$ haplotype characterized by private *H-2* antigens *H-2K.19* (detected by antiserum K-119) and *H-2D.4* (detected by antiserum K-304), and by Ia antigens Ia.3 and 4 (detected by antiserum K-212). Genetic mapping indicates that our antigens Ia.3 and 4 are controlled either by the *IA* or *IB* subregion of the *I* region (7). The table shows that pretreatment of B10.HTT spleen cells with anti-*H-2D.4* induces resistance to anti-*H-2D.4* in the presence of complement, but not to anti-*H-2K.19* nor to anti-Ia.3, 4; pretreatment with anti-*H-2K.19* induces resistance to anti-*H-2K.19*, but not to anti-*H-2D.4* nor to anti-Ia.3,4; and finally, pretreatment with anti-

TABLE V

Susceptibility of B10.HTT Spleen Cells To Cytotoxic Killing After Anti-H-2 or Anti-Ia and RAMIG Treatment

Pretreated with:	Tested with:	Dead cells at dilution of As:		
		1:4	1:8	1:16
		%	%	%
—	K-304	95	70	30
K-304(1:2) + RAMIG(1:4)	K-304	38	35	30
K-304(1:2) + RAMIG(1:4)	K-119	95	60	28
K-304(1:2) + RAMIG(1:4)	K-212	55	52	55
—	K-119	60	60	48
K-119(1:4) + RAMIG(1:4)	K-119	30	28	28
K-119(1:4) + RAMIG(1:4)	K-304	95	60	30
K-119(1:4) + RAMIG(1:4)	K-212	52	55	50
—	K-212	55	55	50
K-212(1:4) + RAMIG(1:4)	K-212	30	30	28
K-212(1:4) + RAMIG(1:4)	K-304	78	75	50
K-212(1:4) + RAMIG(1:4)	K-119	55	58	55
RAMIG(1:4)	NMS + C′	30	28	25

Ia.3,4; induces resistance to anti-Ia.3,4 but not to anit-*H-2K.19* nor to anti-*H-2D.4*. Thus, all three antigenic moieties appear to be distinct entities in the cell membrane. The AQR strain (Table VI) carries the $H\text{-}2^{y1}$ haplotype characterized by private antigens *H-2K.17* (detected by antiserum K-26) and *H-2D.4* (detected by antiserum K-304), and Ia antigen 1 (detected by antiserum K-210), controlled by the IA subregion of the *I* region (17). In all combinations pretreatment with one antiserum induced resistance to the same antiserum but not to the other two antisera, thus confirming the molecular independency of the *H-2K*, *H-2D*, and Ia products.

In some cases, the pretreatment with one antiserum actually increased the susceptibility to the second or the third antiserum. Such an increase was particularly noticeable when the test antiserum was against the Ia antigens. It often killed up to 80% of the target cells pretreated with an anti-*H-2* serum (compared to a maximum of 60% killing of untreated cells). Table VII shows that the increase is not caused by selective elimination of one type of lymphocytes (T cells) during the pretreatment procedure: the percentage of spleen cells killed by anti-Thy-1.2 and complement following the pretreatment is approximately the same as in untreated cell suspension. The possibility that under these circumstances the anti-Ia react not only with B cells but also with some T cells, is being investigated.

Molecular Independency of Ia, H-2, and Ig Antigens. Since the lysostrip technique uses anti-Ig antibodies as a second layer, and since splenic B cells are known to carry Ig receptors on their surfaces, it was necessary to demonstrate that the Ig receptors are not physically linked to either *H-2* or Ia molecules. Table VII shows that this is not the case, indeed. Pretreatment of B10.HTT spleen cells with anti-Ig sera did not reduce cytotoxic killing in the presence of complement by either *H-2* or Ia sera. In this experiment, the efficiency of stripping of B cells of

TABLE VI

Susceptibility of AQR Spleen Cells to Cytotoxic Killing After Anti-H-2 or Anti-Ia and GAMIG Treatment

Preatreated with:	Tested with:	Dead cells at dilution of As:		
		1:4	1:8	1:16
		%	%	%
—	K-304	60	50	25
K-304(1:2) + GAMIG(1:4)	K-304	15	15	12
K-304(1:2) + GAMIG(1:4)	K-26	90	60	40
K-304(1:2) + GAMIG(1:4)	K-210	60	60	25
—	K-26	95	85	25
K-26(1:8) + GAMIG(1:4)	K-26	35	25	25
K-26(1:8) + GAMIG(1:4)	K-304	95	75	25
K-26(1:8) + GAMIG(1:4)	K-210	80	75	50
—	K-210	50	50	30
K-210(1:2) + GAMIG(1:4)	K-210	20	22	20
K-210(1:2) + GAMIG(1:4)	K-304	90	70	40
K-210(1:2) + GAMIG(1:4)	K-26	90	80	20
GAMIG(1:4)	NMS + C′	15	20	15

TABLE VII

Susceptibility of B10.HTT Spleen Cells to Ia-, H-2-, and Thy-1-mediated Cytotoxic Killing After Treatment with GAMIG

Pretreated with:	Tested with:	Dead cells at dilution of As:		
		1:4	1:8	1:16
		%	%	%
—	K-212	48	35	20
GAMIG(1:4)	K-212	45	32	20
—	K-304	95	95	80
GAMIG(1:4)	K-304	80	80	80
—	K-119	75	50	45
GAMIG(1:4)	K-119	75	55	35
—	K-226	35	35	28
GAMIG(1:4)	K-226	50	50	50
GAMIG(1:4)	NMS + C′	18	15	12

their Ig receptors was controlled by staining the cells with fluoresceinated anti-Ig serum. In the sample that was not pretreated with GAMIG, between 32–40% of the cells were stained with fluoresceinated RAMIG. After pretreatment with GAMIG (2 h at 0°C and 30 min at 37°C), the percentage of cells stained with fluoresceinated RAMIG was reduced practically to 0.

Discussion

The studies described in this communication demonstrate that, using the lysostrip method and monospecific *H-2* and Ia antisera, specific resistance to antibody and complement-mediated cytotoxicity can be induced. The resistance

is always restricted to the genetic product of one of the three *H-2* regions (*K*, *I*, and *D*), namely to the one with which the antibodies in the particular antiserum reacted; the products of the other two regions remain susceptible to antibody-complement attack. Furthermore, in the B cells, the removal of the Ig receptors from the cell surface by anti-Ig treatment does not significantly reduce reactivity of these cells with anti-Ia, anti-*H-2K*, or anti-*H-2D* sera. This observation suggests that antigens controlled by the *K*, *I*, and *D* regions are present on separate molecules moving independently in the cell membrane, and that at the same time, the three types of molecules move independently of the Ig molecules in B cells.

These results are in agreement with those of biochemical studies, and in the case of *H-2* antigens, also with the results of the study on fluoresceinated antibody-induced antigen redistribution. Cullen and her co-workers (19) were the first to demonstrate that the private antigens controlled by the $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes behaved as independent units after chemical separation. The authors labeled $H\text{-}2^b$ antigens with [^{3}H]fucose, solubilized them with NP-40 detergent, and reacted the soluble material with anti-*H-2.2* antibodies in an indirect immunoprecipitation assay. After removal of the precipitate, they reacted the supernate with anti-*H-2.33* serum and found that the pretreatment removed all the *H-2.2* activity but did not affect the *H-2.33* activity. They also demonstrated similar independent behavior in all other combinations of the four private $H\text{-}2^b$ and $H\text{-}2^d$ antigens.

Our results closely parallel those obtained by Neauport-Sautes and her colleagues (5) using the fluoresceinated-antibody-induced antigen redistribution method. The authors incubated $H\text{-}2^b$ lymphocytes with tetramethylrhodamine-isocyanate-conjugated anti-*H-2.33* at 37°C, then cooled the cells to 0°C and tested the cells with fluorescein-isothiocyanate-conjugated anti-*H-2.2* antibodies. They observed that after such treatment the red fluorescence corresponding to antigen *H-2.33* was accumulated in caps at one pole of the cells, whereas the green fluorescence corresponding to antigen *H-2.2* remained diffused, clearly separated from the red cap. Thus, antigens controlled by one *H-2* region (e.g., *K*) can be redistributed independently of antigens controlled by another *H-2* region (*D*).

Molecular individuality of Ia antigens was suggested by biochemical studies (15, 16) which demonstrated that immunoprecipitation of NP-40 solubilized material with *H-2* antibodies removes *H-2*, but not Ia activity, and vice versa. Our data represent the first demonstration of independent behavior of *H-2* and Ia antigens and Ia and Ig antigens in the intact cell membrane. Thus, the technique of induction of resistance to antibody and complement-mediated cytotoxicity is promising to be a very useful tool for studying the molecular interrelationships in a complex antigenic system.

The mechanism of the resistance induction is largely unknown. It was originally believed that the combined allo- and xenoantibody pretreatment completely stripped the membrane of the antigens against which the pretreatment alloantiserum is directed, and that this stripping is achieved by aggregation of the antigens, by their capping and by the disappearance of the aggregates and caps from the cell membrane by pinocytosis and/or exfoliation. However, more recent observations argue against such a simplistic explanation. It was reported, for example, that cells that are specifically resistant when tested in

the presence of the guinea pig complement are susceptible to lysis when rabbit complement is added (20). This observation would seem to suggest that, rather than being removed from the cell surface, the antigens are transformed into a state in which they are inaccessible to the action of the guinea pig complement.

The mechanism of the resistance induction is an interesting question that deserves a detailed analysis. However, no matter what the mechanism is, it is clear from the studies of Bernoco et al. (3), Cullen et al. (4) and from the studies reported here that the procedure has a high degree of specificity and that it can be, therefore, used in lieu of the fluoresceinated antibody-induced antigen redistribution method. The resistance-induction method has several advantages over the fluorenceinated antibody method. The resistance induction method is less time consuming, since it does not require laborious labeling of the alloantibodies; it avoids the reduction in antibody titres caused by the conjugation procedures; and it does not require any special optical equipment. The technique should be useful in determining, for example, the molecular interrelationships among the various private and public *H-2* antigens, and the relationship among the individual Ia antigens. Experiments along these lines are in progress.

Summary

Mouse spleen or thymus lymphocytes incubated with monospecific *H-2* or Ia alloantisera and then coated with a xenogeneic antimouse Ig serum become specifically resistant to the alloantiserum (and complement) they have been incubated with. This so called "lysostrip method" was used to investigate the molecular interrelationships of antigens in the mouse lymphocyte membrane. The results of this investigation confirm that *H-2K* and *H-2D* antigens are carried by two distinct populations of molecules. They provide evidence that the Ia antigens move in the membrane independently of both *H-2K* and *H-2D* antigens; and finally they demonstrate absence of any physical linkage between Ig receptors in B cells, on the one hand, and Ia, *H-2K*, and *H-2D* molecules on the other hand.

We gratefully acknowledge the excellent technical assistance of Ms. Jan Pickett and secretarial assistance of Ms. JoAnne Tuttle. We thank Dr. Ellen S. Vitetta for providing goat antimouse Ig sera, to Dr. James Forman for fluoresceinated anti-Ig sera, and to Prof. Ruggiero Ceppellini for advice regarding the human lysostrip technique.

Received for publication 12 December 1974.

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