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ORIGINAL ARTICLE

Navin Reddy · Gaik Lin Ong · Thomas M. Behr
Robert M. Sharkey · David M. Goldenberg
M. Jules Mattes

Rapid blood clearance of mouse IgG2a and human IgG1 in many nude and *nu*/+ mouse strains is due to low IgG2a serum concentrations

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Abstract We reported previously that the blood clearance of injected mouse IgG2a was extremely rapid in many strains of nude and *nu*/+ mice. In an attempt to determine the cause of this phenomenon, the levels of endogenous IgG2a in the blood of these mice was assayed. It was found that the serum level of IgG2a was extremely low in many of these mice, below 50 μg/ml, which is 20–100 times lower than the expected normal value. Great heterogeneity between individual mice was observed in their blood level of IgG2a, and there was an excellent correlation between low blood IgG2a levels and rapid clearance of injected IgG2a. Thus, the blood IgG2a levels are so low that a novel, previously undescribed effect occurs, namely the rapid clearance of small amounts of injected IgG2a. The clearance is due primarily to binding sites in the spleen and liver. The low level of endogenous IgG2a is not due to the lack of a thymus, since it occurs in *nu*/+ as well as nude mice, but can probably be attributed to the very clean environment in which these mice are raised. In assays of sera from approximately 50 mouse strains, low IgG2a levels were found in all nude colonies and also in some normal mouse strains. Some nude mice displayed relatively normal IgG2a clearance rates despite having low levels of endogenous IgG2a. In repeated bleedings of individual mice, IgG2a levels were found to fluctuate greatly. A similar clearance effect was observed with a human IgG1 Ab injected into mice. This rapid clearance of injected IgG, of certain subclasses, represents a practical problem for many experiments in which antibodies are used for diagnosis or therapy, and several methods of circumventing the problem are discussed.

N. Reddy · G.L. Ong · T.M. Behr[1] · R.M. Sharkey · D.M. Goldenberg · M.J. Mattes (✉)
Garden State Cancer Center at the Center for Molecular Medicine and Immunology, 520 Belleville Avenue, Belleville, NJ 07109, USA

Present address:
[1] Department of Nuclear Medicine, Georg-August-University of Göttingen, D-37075, Germany

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Introduction

In attempts to utilize antibodies for therapy, the blood clearance of the injected Ab is a basic parameter that will strongly affect the results obtained. The blood clearance $t_{1/2}$ for mouse IgG2a, in normal mice, is approximately 4 days [4, 5, 16]. Mouse IgG2a is one of the major IgG subclasses [5, 16], present at a serum concentration of more than 1.0 mg/ml in adult mice, and is the most potent of the IgG subclasses in biological effector functions, including complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. We reported previously that, unexpectedly, the clearance of injected IgG2a from the blood of nude and *nu*/+ mice was extremely rapid [20]. Such clearance presented a major obstacle for our purpose, which was to assay the localization of Ab to tumors growing s.c. in these mice. That is, most of the Ab was cleared from the blood before it could reach the intended target, the tumor. The major observations were the following. (1) Rapid IgG2a clearance occurred in both nu/nu and *nu*/+ mice from all major suppliers tested, but did not occur in +/+ mice of the same outbred strains. (2) Rapid clearance did not occur in BALB/c nude mice. (3) Mice were markedly heterogeneous in this regard, with mice from the same lot showing either normal or fast clearance rates. (4) Both IgG2a and the closely related IgG2b were affected, while IgG1 was not. (5) Rapid clearance was dependent on the Fc region of the Ab, and was due primarily to uptake by the liver and spleen. (6) Rapid clearance could be blocked by increasing the dose injected, and 0.1 mg/mouse was found sufficient to block rapid clearance for 3 days. Similar observations were recently reported by Reist et al. [18].

The explanation suggested for these results, now known to be incorrect, was based on the assumption that *nu*/+ mice have normal, high blood levels of IgG2a. Thus, it was necessary to postulate that the IgG2a injected, which were

BALB/c monoclonal Ab, were different from the IgG2a present in the blood of the mice injected. Also, since *nu*/+ but not +/+ mice displayed rapid IgG2a clearance, we suggested that this might be a dominant affect of the nude gene, even though such dominant effects have not been described previously [19]. We herein describe further experiments that disprove our original assumptions, and demonstrate that IgG2a clearance is, instead, correlated with the level of endogenous IgG2a present in the blood.

Materials and methods

Animals and mouse sera

The mice used were obtained from Harlan Sprague Dawley (Indianapolis, Ind.), Taconic Farms (Germantown, N.Y.), or Jackson Laboratories (Bar Harbor, Me.). NFS and FVB inbred nude and *nu*/+ mice were obtained from Dr. Carl Hansen at the National Insitutes of Health (Bethesda, Md.). Unless noted, the age and sex of the mice used were as follows: HSD *nu*/+, 6.5-week-old females; HSD nude, 6.5-week-old males; Taconic Swiss *nu*/+ and nude, 8.5-week-old females; Taconic NCr *nu*/+, 6-week-old females; Taconic NCr nude, 8.5-week-old females; Jackson NU *nu*/+ and nude, 9-week-old males; Jackson BALB/c *nu*/+ and nude, 8-week-old females; BALB/c (Taconic), 11-week-old females. The mice designated *nu*/+ were all definitely *nu*/+, except for the Jackson BALB/c nudes, which are really ?/+, such that 2/3 are *nu*/+ and 1/3 +/+. Both nude and *nu*/+ mice were maintained, in the same room, in a Thoren caging system in which individual filtered cages are maintained at a positive air pressure. Food was autoclaved and water was sterile (for irrigation, Abbott Labs, North Chicago, Ill.), and supplemented with penicillin V (VEETIDS for oral solution, Bristol-Myers Squibb, Princeton, N.J.) to 0.67 mg/ml. Other mouse sera tested for their IgG2a content were previously described [13, 14], except that the HORT-D serum was from Dr. Verne Chapman (Roswell Park Memorial Institute, Buffalo, N.Y.). These sera were all from male mice, mostly pooled from 2–3 mice 8–16 weeks old, except as noted previously [14].

Antibodies and radiolabeling

The IgG2a Ab MA103 and LL2 and the humanized monoclonal Ab hu-LL2, having the human IgG1 heavy chain, were described previously [10, 20]. Ascites of another IgG2a, UPC10, was obtained from Sigma Chemical (M7769; St. Louis, Mo.). Ab were iodinated with ^{125}I as previously described [20], and more than 90% of the radioactivity migrated with IgG on an HPLC gel-filtration column.

Determination of the blood clearance rate of IgG2a, and serum collection

These methods were described previously in detail [20]. Briefly, 10^6 cpm ^{125}I was injected i.v. and bleedings obtained at various times, as indicated in the text. Inhibitory Ab were mixed with the radiolabeled Ab before injection. The first bleeding was obtained immediately after injection (approximately 30 s). For the last bleeding, usually at 24 h, mice were exsanguinated. After the blood had been allowed to clot, serum was collected and stored at –70 °C until it was assayed for IgG2a content.

Enzyme-linked immunosorbent (ELISA) assay for mouse IgG2a

Nunc 96-well plates (Maxisorp Immuno-Plates, VWR Scientific, N.J.) were coated overnight at 4 °C with 0.1 ml goat anti-(mouse IgG2a) (GAM/IgG2a/7S, Nordic Labs, Capistrano Beach, Calif.) at 10 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6. Plates were washed three times with phosphate-buffered saline (PBS)/0.05% Tween 20 (Sigma), then incubated with 0.2 ml PBS/1.0% bovine albumin/10 mM NaN_3. After 1 h at 37 °C, plates were washed three times with PBS/Tween, and a 0.1-ml sample of a serum dilution was added. Serum was diluted in PBS/10 mM NaN_3/5% rabbit serum (Normal carrier serum, Pelfreez, Rogers, Arkansas). All sera were initially tested at 1:100, 1:400 and 1:1600. For sera that were found to have an IgG2a concentration that exceeded the limit of the assay (above 960 µg/ml), a second assay was performed with tenfold higher dilutions. After 2 h at 37 °C, plates were washed six times with PBS/Tween, and 0.1 ml peroxidase-conjugated goat anti-(mouse Fab) (GAM/Fab/PO, Nordic Labs) was added at a 1:1000 dilution in PBS/1.0% bovine albumin/0.1% bovine immmunoglobulin (Sigma)/0.01% Tween-20/0.05% thimerosal. After 1 h at 37 °C, the plate was washed six times with PBS/Tween, and 0.1 ml substrate was added, namely *o*-phenylenediamine (Sigma) at 0.8 mg/ml in 0.15 M citrate/phosphate buffer, pH 5.0/0.012% H_2O_2. After 20 min at room temperature in the dark, 0.05 ml 2 M H_2SO_4 was added, and the absorbance of the wells read at 490 nm on an ELISA plate reader. Standards used were threefold serial dilutions of LL2, starting at 600 ng/ml, which were included on every plate. A typical standard curve is shown in Fig. 1. IgG2a concentrations of the test samples were calculated by interpolation from the standard curve; since the curve flattened out above 200 ng/ml, only the portion of the curve from 2.5 ng/ml to 200 ng/ml was used. Values calculated from the three dilutions of the test samples were consistent, and the means of these values are shown here. Each plate also included a medium control and a mouse serum control, which was a mouse serum selected to have a moderate IgG2a concentration (50–100 µg/ml), and was used to monitor the day-to-day consistency of the assay. The mean value obtained with this standard, for the assays performed in this study, was 68.8 ± 9.2 µg/ml. All tests were done in duplicate, and variations between the duplicates were small compared to variations between samples.

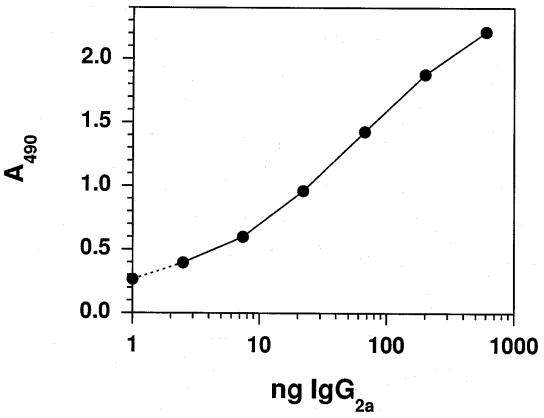

Fig. 1 Representative standard curve of enzyme-linked immunosorbent assay (ELISA) for mouse IgG2a

Results

The effect of the IgG2a allele on the rapid blood clearance of IgG2a

As described above, we initially assumed that there must be some difference between the IgG2a-injected (BALB/c monoclonal Ab) and the IgG2a present endogenously in the blood of the mice injected. There are 12 known alleles of mouse IgG2a [15], coded for by the genetic locus *Igh-1*. Accordingly, we attempted to identify an allele that was

similar to that present in the nude mice, which were outbred Swiss. The approach was to use sera from various mouse strains, selected for their known IgG2a alleles, to inhibit rapid blood clearance of radiolabeled BALB/c IgG2a mAb. In preliminary experiments, it was found that 0.1 ml BALB/c serum (*Igh-1a*), mixed with the radiolabeled Ab, was sufficient to block rapid clearance. Accordingly, inhibition was tested with sera from the mouse strains BUB, C57BL/6, DBA/2, A/WySn, CE, DA/HuSn, SEA/Gn and PL, representing the IgG2a alleles *a,b,c,e,f,g,h,* and *j* respectively. Our prediction was that sera from the "correct" allele would not inhibit rapid clearance. However, all sera tested were found to inhibit clearance effectively (data not shown).

Another approach was to directly identify an inbred nude strain that displayed rapid clearance. NFS and FVB *nu*/+ mice were selected because they were derived from Swiss outbred mice. *nu*/+ mice were tested because they have demonstrated more consistent rapid clearance than nu/nu mice. However, rapid blood clearance did not occur in these mice (data not shown).

Given these negative results, it appeared that clearance might not be dependent on the IgG2a allele. Indeed, we previously reported that rapid blood clearance appeared to be less common in older mice [20]; this might be due to increased production of IgG2a in these mice, although other explanations are also possible. In order to demonstrate directly that outbred nude strains are able to synthesize an IgG2a, which is, for our purposes, the same as a BALB/c IgG2a, older HSD *nu*/+ mice, 8 months old, were selected that displayed slow IgG2a clearance, and sera from these mice were tested for their ability to block rapid blood clearance in young, 6- to 8-week old HSD *nu*/+ mice. In fact, effective inhibition was observed (data not shown). These results together indicated that HSD nude and *nu*/+ mice *could* synthesize IgG2a that was able to block rapid clearance, and that younger mice frequently did not produce enough IgG2a to have this effect. Since 100 μg purified IgG2a effectively blocked clearance, the level of endogenous IgG2a in 6- to 8-week-old mice must be less than approximately 50 μg/ml.

ELISA assays for mouse IgG2a in HSD nude and *nu*/+ mice, and correlation with rapid blood clearance of IgG2a

On the basis of the above results, the IgG2a concentration in sera of individual mice of various strains was determined. The ELISA assay could detect 2.5 ng/ml reliably, which was close to the minimum detectable level. All mice tested had measurable IgG2a. In control experiments, IgG1 mAb were totally negative in this assay. Since most nude mouse sera had very low IgG2a levels, as shown below, an additional control was used to determine whether nude mouse serum contained an inhibitory factor that might interfere with the assay. For this purpose, purified IgG2a was mixed with a nude mouse serum selected for apparent low IgG2a content: no inhibitory factor was detected.

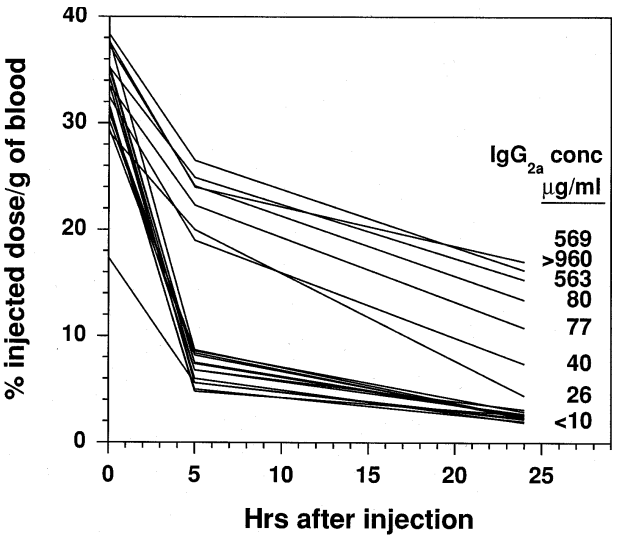

Fig. 2 Correlation between IgG2a concentration in serum and clearance of radiolabeled IgG2a in HSD nude mice. Each line represents data from an individual mouse. After clearance of radiolabeled IgG2a had been monitored for 24 h, the mice were bled out, and the IgG2a concentration determined in an ELISA assay. The IgG2a concentration corresponding to each individual mouse is listed on the right

Initially, 20 HSD nude mice, 6.5 weeks old, were tested, since these mice have consistently displayed heterogeneity in their IgG2a clearance rates. Such heterogeneity is an advantage in demonstrating a correlation between IgG2a clearance rate and the level of endogenous IgG2a in blood. In fact, the 20 nude mice tested displayed the expected striking heterogeneity in their blood concentration of IgG2a, and this was correlated with the clearance of injected IgG2a (Fig. 2). Of the 20 mice, 13 had IgG2a levels below 10 μg/ml, and all these mice showed rapid clearance. Three mice had more than 560 μg/ml IgG2a, and these mice displayed slow clearance. The remaining 4 mice had intermediate IgG2a levels, ranging from 26 μg/ml to 80 μg/ml; these mice showed intermediate clearance rates, and the clearance rate was perfectly correlated with the serum concentration of IgG2a (Fig. 2). A serum concentration of approximately 80 μg/ml was sufficient to produce relatively normal IgG2a clearance, while 30–40 μg/ml produced partial inhibition of rapid clearance. These data indicate that, at least in this mouse strain, rapid IgG2a clearance is due to lack of sufficient IgG2a in the blood, and that approximately 100 μg/ml is required to produce normal clearance.

HSD *nu*/+ mice were also tested, for comparison. In the earlier studies, these mice showed more consistent rapid blood clearance than the HSD nude mice, with virtually every mouse having rapid blood clearance. In tests of 10 mice, this was confirmed. (The mean clearance rate is included in Fig. 7.) The IgG2a concentration in the sera of these mice was consistently very low, with little heterogeneity between mice (Fig. 3): 7.0 ± 2.4 μg/ml.

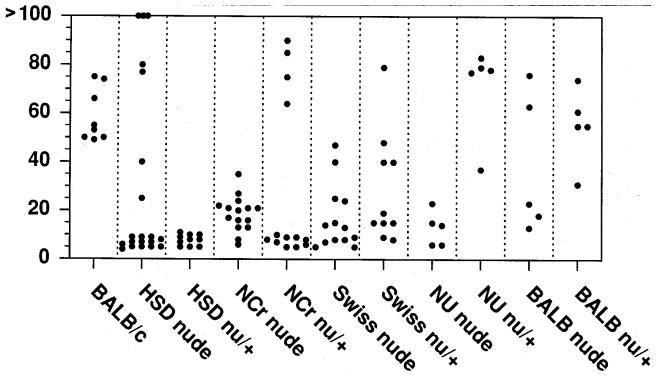

Fig. 3 Serum IgG2a concentration in mice of various strains. Each point represents data from an individual mouse. The age and sex of the mice tested are described in Materials and methods

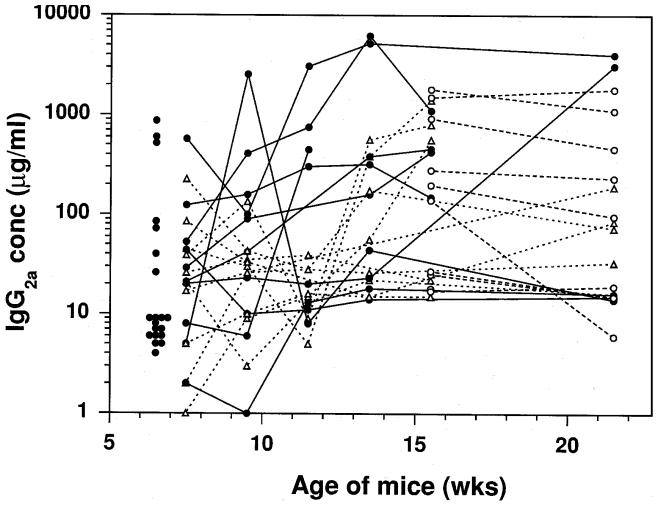

Fig. 4 Serum IgG2a concentration in HSD nude mice bled repeatedly, from 6.5 to 21.5 weeks of age. Each curve represents data from an individual mouse. The points on the left represent data from mice that were bled only once. Different symbols and line types are used solely to facilitate identification of separate lines. IgG2a concentrations varied greatly between individual mice, and also varied greatly between different bleedings of the same mouse

Serial bleedings of individual HSD nude mice

One of the peculiar characteristics of this experimental situation is the heterogeneity between individual nude mice. It was therefore of interest to determine whether mice would have consistently high or low IgG2a levels when tested over a period of months. The heterogeneity between mice may be, in a sense, easier to explain if IgG2a levels fluctuate. Therefore, individual mice were bled repeatedly, from 7 to 22 weeks of age, and the sera tested for IgG2a concentration. The IgG2a concentrations fluctuated greatly in individual mice (Fig. 4). While there was a pronounced trend for later bleedings to have higher IgG2a levels, some individual mice showed a large decrease in IgG2a concentration with time. These results are consistent with our earlier observations that older HSD/nude mice were less likely to display rapid IgG2a blood clearance [20]. To confirm further the relationship between endogenous IgG2a concentration and clearance of injected IgG2a, 9 of the 15.5-week-old mice, selected for their high serum IgG2a concentration, were tested with an injection of radiolabeled IgG2a. As expected, all of these mice displayed slow clearance.

The IgG2a concentration in 7.5-week-old mice ranged from 1 μg/ml to 573 μg/ml, and in 13.5-week-old mice the range was 14 μg/ml–6.2 mg/ml, a 440-fold range. It is interesting to compare these results with the earlier experiment, described above, in which the mice (from the same batch) were 1 week younger, 6.5 weeks of age. These data are also included in Fig. 4, and show that the IgG2a concentration increased significantly from 6.5 to 7.5 weeks of age. Very low IgG2a concentrations, below 10 μg/ml, were not detected in nude mice older than 13.5 weeks. However, some of the older mice still displayed IgG2a concentrations low enough for rapid blood clearance of injected IgG2a to be expected. Thus, 7 of 17 mice tested at 13.5 weeks had IgG2a levels of less than 27 μg/ml, and at least 4 mice at 21.5 weeks had IgG2a levels of less than 20 μg/ml. Despite the overall upward trend in IgG2a concentration with age, at least 4 mice showed marked decreases with time, such that the IgG2a levels changed from above 100 μg/ml (a level at which normal IgG2a clearance would be expected), to less than 50 μg/ml (a level at which rapid clearance would be expected).

Human IgG1 blood clearance in mice

Preliminary experiments suggested that humanized Ab, containing the human IgG1 heavy chain, were cleared from the blood of mice similarly to mouse IgG2a. This would be consistent with the fact the mouse IgG2a and IgG2b are functionally homologous to human IgG1 [21], although the genetic relationship between the IgG subclasses of different species is complex [2, 7]. To test this possibility, HSD *nu*/+ mice were injected with radiolabeled hu-LL2. The Ab was in fact cleared quite rapidly in 10 of 11 mice (Fig. 5A). In only 3 of the mice was clearance as fast as that of a mouse IgG2a; the other 7 mice showed an intermediate clearance rate. To demonstrate that the hu-IgG1 was cleared via the same mechanism as mouse IgG2a, two approaches were used. First, the IgG2a level in the sera of these 11 mice was assayed; as predicted, the 1 mouse with slow clearance had the highest concentration of endogenous IgG2a, 54 μg/ml. The other 10 mice had IgG2a levels in the range 4–16 μg/ml. We note that this experiment demonstrates that HSD *nu*/+ mice can occasionally have substantial levels of IgG2a, but this is rare (1 of 21 mice in this study, and similarly rare in previous experiments [20]). The second approach was to perform reciprocal competitive inhibition experiments, with mouse IgG2a and human IgG1. Mouse IgG2a effectively inhibited the clearance of hu-LL2 (data not shown). Moreover, hu-LL2 was able to inhibit the clearance of mouse IgG2a, although it was less potent than mouse IgG2a (Fig. 5B).

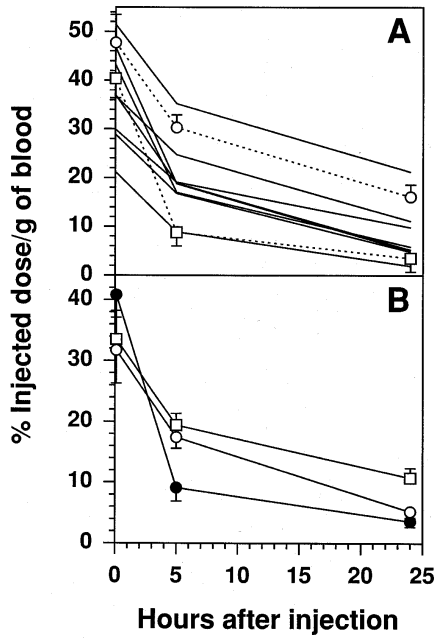

Fig. 5A, B Human IgG1 is cleared from blood by the same mechanism as mouse IgG2a. **A** Clearance of hu-LL2 from the blood of HSD *nu*/+ mice. Each solid line represents data from an individual mouse. Also shown, for comparison, are results obtained with BALB/c mice (a control having normal clearance; ○), and HSD *nu*/+ mice (a control having consistent rapid clearance; □). For these control curves, shown by dashed lines, means ± standard deviations are shown. **B** Rapid blood clearance of radiolabeled mouse IgG2a is inhibited by human IgG1. HSD *nu*/+ mice were injected with radiolabeled mouse IgG2a (●). Some mice were also injected with 0.2 mg human IgG1 (hu-MN-14; ○) or 0.1 mg mouse IgG2a (□). Results shown are means ± standard deviations for groups of at least 4 mice. Human IgG1 inhibited rapid IgG2a clearance, but not as effectively as mouse IgG2a

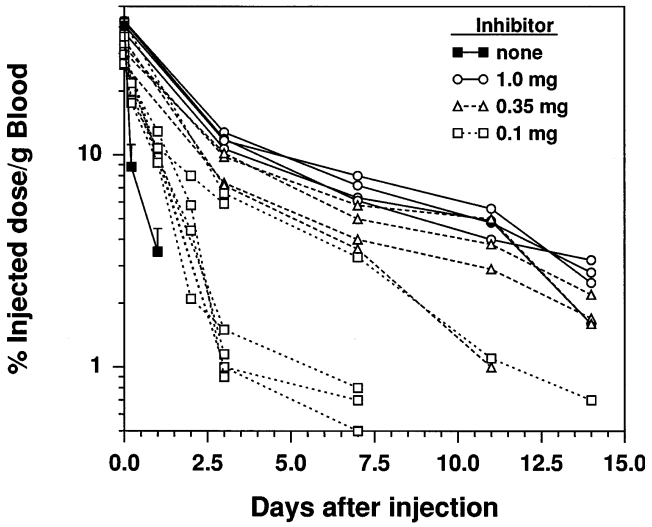

Fig. 6 Inhibition of rapid blood clearance of IgG2a by higher protein doses, over a 2-week period. HSD *nu*/+ mice were injected with radiolabeled IgG2a together with varyious amounts of unlabeled IgG2a (UPC10), as indicated. Each line shows results obtained with an individual mouse, except for the control curve, obtained in the absence of an inhibitor, which shows means ± standard deviations. A 1.0-mg sample of IgG2a produced essentially complete inhibition of rapid clearance, with IgG2a blood clearance similar to that observed in BALB/c mice (not shown); 0.35 mg IgG2a produced fairly strong but incomplete inhibition; 0.1 mg produced only partial inhibition, which was observed at early assay times (primarily less than 1 day), but was ineffective at day 3 or later

These results are consistent with the interpretation that mouse IgG2a is the homologous protein in mice, and that human IgG1 shows partial cross-blocking of the clearance mechanism.

Inhibition of rapid IgG2a clearance by increased protein dose

We previously reported that injection of 100 μg IgG2a was sufficient to prevent rapid clearance from blood. However, those experiments only lasted 3 days. Since typical Ab biodistribution experiments extend for 14 days, it was important to determine whether IgG2a clearance could be blocked for this period of time. Also, we wanted to test a different source of non-specific IgG2a, UPC10 ascites from Sigma Chemicals, since purified Ab should not be necessary for this purpose, and since we hoped to identify an inexpensive source of IgG2a that could be used in hundreds of mice. The concentration of UPC10 in the ascites was determined by our ELISA method, and results were consistent with the product information provided by the supplier. As shown in Fig. 6, UPC10 was able to inhibit rapid clearance of IgG2a, but a large amount, 1.0 mg/mouse, was required to achieve effective inhibition for 14 days. A dose of 0.1 mg/mouse produced fairly effective inhibition for 1 day, but by day 3 this inhibition was poor. This 0.1-mg dose appeared to be more effective in the previous study [20], perhaps because of the difference in the mice used (nude rather than *nu*/+).

Tests of other strains of nude mice

One of the major purposes of these experiments was to identify a method by which rapid IgG2a clearance can be circumvented. It was found previously that BALB/c nude mice did not display rapid clearance, although only a small number were tested. Since this difference in BALB/c nude mice was *not* due to differences in the IgG2a allele, as discussed above, it seemed possible that an outbred nude strain could be identified that would not display rapid clearance. Outbred nude mice have significant advantages over BALB/c nude mice in terms of hardiness, availability and cost. We had previously tested outbred nude mice from Charles River Laboratories and the Memorial Sloan-Kettering animal colony, as well as HSD, and found that rapid IgG2a clearance occurred in all, although with varying frequency. Therefore, we tested additional nude strains, from Taconic Farms and The Jackson Laboratory. Both nude and *nu*/+ mice were tested, since, with HSD mice, the *nu*/+ mice show more consistent rapid IgG2a clearance than nude mice. Taconic normal BALB/c mice were also included as a control. The mice were tested for both IgG2a clearance and blood level of endogenous IgG2a.

As shown in Figs. 3 and 7, there were considerable differences between strains, with each strain displaying certain unique characteristics. In general, there was frequently a strong correlation between IgG2a blood level and the clearance of injected IgG2a, but some apparent exceptions were also noted, suggesting that IgG2a clearance could be slow in some mice despite a low level of serum IgG2a. We speculate that the clearance mechanism itself may operate at various levels in different mouse strains. The only nude strain showing normal IgG2a clearance was Jackson BALB/c. Jackson NU and Taconic Swiss nude mice showed slightly accelerated IgG2a clearance, yet the clearance rate was probably slow enough, and consistent enough, to be acceptable for most purposes: the blood level at 24 h was $11.6 \pm 0.9\%$ and $12.2 \pm 1.8\%$ of the injected dose/g, respectively, for these two strains.

Other differences between strains should also be noted. (1) None of the other strains had individuals with very high IgG2a concentrations, as was characteristic of a few of the HSD nude mice. The three HSD nude mice with IgG2a levels shown as above 100 μg/ml in Fig. 3, in fact, had levels of 520–869 μg/ml; in contrast, the highest value obtained for any of the other mouse strains tested (in Fig. 3) was 90 μg/ml. (2) The Taconic normal BALB/c mice had low levels of IgG2a, 59 ± 11 μg/ml, which is far below the level usually considered normal, and probably just enough to provide normal clearance rates for injected IgG2a. (3) Some but not all strains displayed two distinct populations of mice, one with very low IgG2a levels, and the other with elevated levels. This was seen clearly in NCr *nu*/+ mice (but not in NCr nude mice), as well as in HSD nude mice (but not in HSD *nu*/+ mice). In such strains, the correlation between IgG2a blood levels and clearance of injected IgG2a was clear. The correlation in HSD nude mice was described above; in NCr *nu*/+, the 4 mice (out of 13) showing slow clearance of IgG2a (Fig. 7B) were the same 4 mice having relatively high blood levels of IgG2a (Fig. 3, fifth column). In contrast, other strains appeared to consist of a single population, albeit with considerable heterogeneity, such as the NCr nude mice; again, this heterogeneity showed up in both assays employed, and there was a clear correlation between the two assays (data not shown).

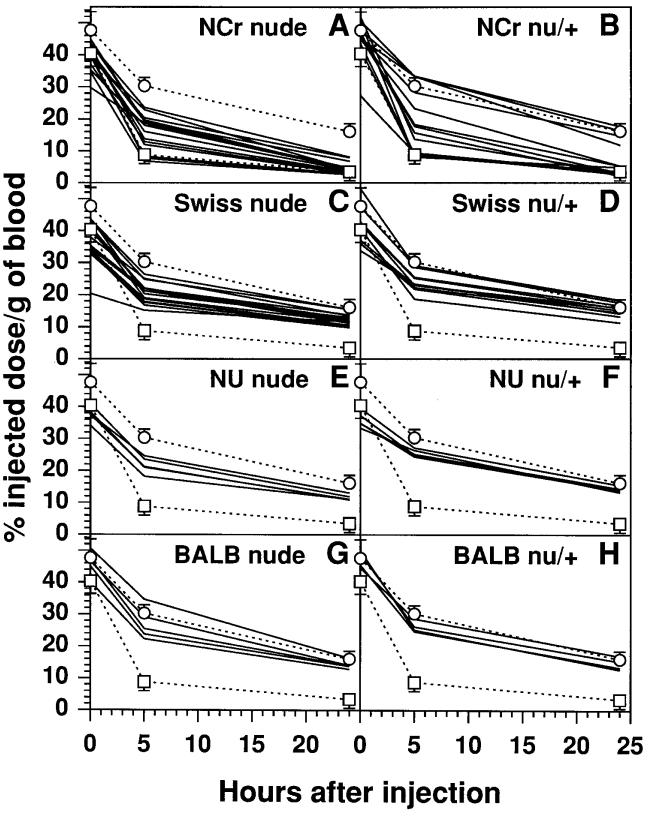

Fig. 7 Blood clearance of radiolabeled IgG2a in nude and *nu*/+ mice of various strains. Each solid line represents data from an individual mouse. Also shown in each panel, for comparison, are results obtained with BALB/c mice (a control having normal clearance; ○), and HSD *nu*/+ mice (a control having consistent rapid clearance; □). For these control curves, shown by dashed lines, means ± standard deviations are shown. The mouse strains tested are indicated

IgG2a serum concentration in normal mouse strains

Normal mice are expected to have high IgG2a levels of more than 1.0 mg/ml, much higher than we observed in either normal BALB/c or most *nu*/+ mice. To evaluate the significance of these results, IgG2a concentrations were assayed in various mouse strains, both inbred and outbred, and including wild and wild-derived mice. This panel of mouse sera, collected for other purposes, primarily consisted of pools from 2–3 male mice, 8–16 weeks of age. As shown in Table 1, mouse strains varied greatly in IgG2a concentration. Many sera had high levels of IgG2a, of above 1.0 mg/ml, but, of the 45 strains tested, 8 had IgG2a levels below 50 μg/ml, at which concentration rapid clearance of injected IgG2a would be expected. Of the 5 Harlan mouse strains tested, 3 had less than 50 μg/ml IgG2a; the other strains with such low levels of IgG2a were 5 of the 29 strains from Jackson.

Discussion

The major conclusion from this study is that many nude and *nu*/+ mice have such low levels of endogenous IgG2a that a small dose of injected IgG2a is cleared from the blood very rapidly, much more rapidly than the normal clearance rate for this Ab. One interpretation is that there are certain binding sites for IgG2a in a mouse, and that these must be occupied before normal clearance mechanisms can function. There are several aspects of these results that warrant discussion.

First, why is the IgG2a level so low in euthymic mice? IgG2a is a thymus-dependent Ab subclass, and thus it would be expected that IgG2a levels might be low in athymic mice. However, the key point of our data is that, in general, the IgG2a levels in *nu*/+ mice were as low as in nu/nu mice. Therefore, it can be concluded that the low

Table 1 IgG2a concentration in sera of various mouse strains

Mouse strain	IgG2a (μg/ml)	Mouse strain	IgG2a (μg/ml)
Jackson		Hansen	
A/WySnJ	180	NFS nude	484
BDP/J	130	NFS *nu*/+	233
BUB/BnJ	2320		
CASA/Rk	851	Harlan	
CAST/Ei	268	C57BL/6	317
DA/HuSn	2492	DBA/2	31
HTG/Go	228	$B6C3F_1$	1745
IS/CamRk	403	$B6D2F_1$	38
LG/J	>9600	$CD2F_1$	7
LP/J	2891		
MA/MyJ	104	Charles River	
MOLF/Ei	446	BDF_1	1751
NZB/B1NJ	897	CD-1	92
NZW/LacJ	29	CF-1	6655
P/J	491		
RBF/DnJ	30	Other wild-derived	
RIIIS/J	2102	CAST	2264
SEA/GnJ	102	CLA	241
SEC/1ReJ	1381	CZECH II	3917
SF/CamEi	47	CZI-O	564
SJL/J	583	HORT-D	1040
SK/CamEi	69	HORT-H	1849
SM-J	1952	HORT-P	357
SWR/J	15	Lewes	602
TIRANO	2292	MOLO	1721
WB/ReJ	366	SKIVE	138
WC/ReJ	311	SPRET-1	706
YBR/Ei	293	WLC-O	468
ZALENDE	24	WSA	153
		WSB	136
		Wild (CO)	2704
		Wild F_1	1073

level of IgG2a is not due to the absence of a functional thymus. The low IgG2a levels can, instead, probably be attributed to the very clean, weakly immunogenic environment in which the mice are raised. This speculation is supported by the results with the 45 other mouse strains tested, since most of these had substantially higher IgG2a levels. The fact that 8 of the 45 strains tested had IgG2a levels below 50 μg/ml can probably be attributed to the clean environment of even normal mouse strains. The strong effect of the "cleanliness" of the environment on IgG2a levels is well known [5, 12], but it is not widely appreciated how low such levels may be in very clean mice. Natsuume-Sakai et al. [12], in 1977, reported very low IgG2a levels in germ-free mice of six strains, consistent with our results. In contrast, Sell and Fahey, in 1965, reported IgG2a levels of 1–2 mg/ml in germ-free mice [5]. It is evident that methods for maintaining nude mice have improved greatly over the past 30 years, that the environment is much cleaner, and that nude mice are much healthier. Thus, the IgG2a concentration in serum has also probably greatly decreased over this period.

Previous studies are not inconsistent with this interpretation. The original measurements of IgG2a levels in nude mice [1, 11] demonstrated that all IgG subclasses were at a much lower level in nude mice than in their *nu*/+ littermates, as expected as a result of the dependence of IgG production on the thymus. However, in these studies, the *nu*/+ mice had reasonably high levels of IgG2a, due presumably to environmental antigen stimulation. Thus, Bloemmen and Eyssen [1] reported mean IgG2a concentrations of 3–4 mg/ml in 6- to 8-week-old *nu*/+ mice. Mink et al. [11] reported mean IgG2a concentrations of approximately 0.9 mg/ml in 6-week-old *nu*/+ mice, and 2.0 mg/ml in 40-week-old *nu*/+ mice. However, Mink et al. stated that their colony was chronically infected with mouse hepatitis virus. It is interesting to note that Mink et al. demonstrated heterogeneity of nude mice similar to that described above: in the 6-week-old mice, approximately 2/3 of the mice had very low IgG2a levels, and the others had high levels, frequently higher than that of *nu*/+ mice.

An established dogma regarding IgG2a clearance is that there is an inverse correlation between blood concentration and clearance rate [8, 23]. That is, high levels induce a faster clearance rate, and low levels a slower clearance rate, and this has been interpreted as a homeostatic mechanism intended to maintain a stable concentration of IgG2a in the blood. Clearly our results contradict this general rule, yet there is no inconsistency in the data, since the lowest IgG2a level tested in these earlier experiments [23] was much higher than the levels observed in the mice tested herein.

What is the mechanism of rapid IgG2a clearance? Previously we demonstrated that the uptake was primarily by the spleen and liver [20]. The mechanism has not been further investigated, but some speculation seems appropriate. CD64, FcγRI, is unique among Fc receptors in that it has a high avidity for monomeric IgG2a [17]. We suggest that this receptor may be responsible for the rapid blood clearance, for the following reasons. (1) It would be expected that FcγRI would bind injected IgG2a, if sites were not already occupied by endogenous IgG2a. Unkeless and Eisen [21] determined the affinity of FcγRI, and calculated that half-saturation of binding sites would occur at an IgG2a concentration of 7 μg/ml. Thus, at the low IgG2a concentrations present in these nude mice, substantial numbers of free binding sites should be present. (2) FcγRI is present on monocytes and macrophages, which is consistent with the spleen and liver localization observed. (3) FcγRI binds IgG2b (weakly) as well as IgG2a, but not IgG1, which is consistent with the specificity of blood clearance. It also would be expected to bind human IgG1: although we are not aware of direct evidence for this, human IgG1 and IgG3 are the subclasses that bind to human FcγRI, and mouse IgG2a binds to human FcγRI [3]. More direct experiments are required to confirm this possible role of FcγRI. If this speculation is correct, these "clean" mice may be useful in experiments on the role of cytophilic Ab on the immune response. It has been suggested that Ab bound to FcγRI on macrophages is important in antigen presentation to lymphocytes [6]. Thus, these mice with low IgG2a levels may allow efficient delivery of injected antigen-specific Ab to the surface of antigen-presenting cells.

Why did rapid clearance of IgG2a not occur in Taconic Swiss, Jackson NU, and Jackson BALB/c mice? Most of these animals, especially the nude mice, had low levels of

endogenous IgG2a, such that rapid IgG2a blood clearance would be expected. We suggest three possible non-exclusive explanations for these results. First, it should be noted that most of these mice did not have the very low endogenous IgG2a concentrations that were seen in the HSD *nu*/+ and most of the HSD nude and NCr *nu*/+ mice. For example, although 6 of 13 Taconic Swiss nudes had IgG2a levels below 10 μg/ml, the other 7 had IgG2a levels above 10 μg/ml. Therefore, it seems conceivable that enough IgG2a is produced to bind to most of the tissue receptors, even though the serum IgG2a remains at a low level. However, this is unlikely considering the excellent correlation between serum IgG2a level and IgG2a blood clearance found in other mouse strains. Second, IgG2b may have played a role. Our earlier study suggested that IgG2b was cleared by the same mechanism as IgG2a, although competition experiments have not been performed. IgG2b binds 20–100 times more weakly to mouse FcγRI than does IgG2a [21, 22]. Thus, it might be predicted that very high levels of IgG2b (independent of IgG2a levels) would effectively inhibit rapid blood clearance. Our decision to focus on IgG2a was based partly on the fact that the IgG2a concentration is generally higher than the IgG2b concentration [5, 16], although this factor also appears to be strain-dependent [12]. The excellent correlation observed in our experiments confirms the adequacy of this decision, perhaps because the IgG2a and IgG2b responses are controlled by similar regulatory factors and are likely to fluctuate together. However, it is possible that certain mice with low IgG2a levels might have higher IgG2b levels, sufficient to block rapid clearance of injected IgG2a. Third, it is clear that there is an additional factor required for rapid IgG2a clearance, in addition to low IgG2a serum levels: there must be an uptake mechanism, although the identity of the mechanism is unknown, as discussed above. Accordingly, it may be that these mice have lower expression of the receptor (possibly FcγRI) that is responsible for IgG2a uptake.

Although we have identified three nude strains that do not display rapid IgG2a blood clearance, it should be emphasized that the great majority of mice used in the United States would be expected to have this characteristic, on the basis of our data. In this report, rapid IgG2a blood clearance was found in HSD and Taconic NCr nude mice (and correlated with low endogenous IgG2a serum concentrations). Our earlier study also reported rapid IgG2a clearance in Charles River (Wilmington, Mass.) nude mice and outbred nude mice from the Animal Resources facility of Memorial Sloan-Kettering Cancer Center (New York, N.Y.). While we have not investigated endogenous IgG2a levels in these last two strains, it is reasonable to assume that the level must be low if rapid IgG2a blood clearance occurs, for the reasons that have been discussed. The three strains that do not show rapid IgG2a blood clearance are all expensive, costing two to four times as much as the other outbred nude mice, and therefore are much less commonly used.

Considering that IgG2a monoclonal Ab are common, and many have been used in nude mouse experiments, why was rapid blood clearance not previously recognized by other investigators? Although we cannot answer this question, two factors should be considered. First, considering the marked variation between individual mice, low blood levels in some mice might be mistakenly attributed to poor i.v. injections. Second, rapid clearance might be attributed to the "normal" activity of Fc receptors, which appears to be the case in the study by Hutchins et al. [9], who used a humanized IgG1 Ab in nude mice.

Rapid IgG2a clearance, and the great heterogeneity between mice in the rate of clearance, constitutes a major obstacle for many experimental purposes [18, 20]. It is clear from Fig. 1 that it would be impossible to obtain reliable data from Ab biodistribution experiments under these conditions. Even if all mice with very rapid blood clearance are excluded, the remaining mice still show marked variability that would obscure the experimental results. Accordingly, it is important to define approaches by which this problem can be circumvented. Injection of large protein doses is effective, but may interfere with some experiments, and is not always practical, considering that approximately 1.0 mg/mouse is required to inhibit rapid clearance for 14 days. The nude mice that display normal or relatively normal IgG2a clearance could be used, but these are all very expensive, as noted above. Of course, the simplest strategy is to use Ab that do not exhibit this rapid clearance, such as mouse IgG1, if possible. A single amino acid substitution in IgG2a can essentially abolish interaction with FcγRI [22], and this modification should be tested for its effect on the type of rapid blood clearance described herein.

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