ABSTRACT

An extremely rapid blood clearance rate of murine IgG2a antibodies was found in all strains tested of outbred Swiss nu/nu mice, including mice from the major commercial suppliers. The clearance half-life was less than 5 h, in comparison to a 4-5-day half-life in BALB/c mice. Therefore, most of the IgG2a antibody injected i.v. in such mice is cleared before it can reach interstitial fluid, which interferes with immunotherapy and immunodetection experiments. Individual nude mice varied greatly in their IgG2a clearance rates, which hampered investigation of the phenomena. In our experience, approximately three-fourths of nude mice had a rapid or intermediate clearance rate, whereas the remainder had an approximately normal clearance rate. The clearance rate in nude mice was age-dependent, at least in some instances, in that a rapid clearance rate was observed at 2 months of age, whereas the same mice retested at 4 months of age had a normal clearance rate. Rapid clearance could be inhibited by increasing the dose injected: 100 µg/mouse resulted in a normal clearance rate, whereas 30 µg/mouse was insufficient to inhibit rapid clearance. The clearance rate of IgG2b antibodies was affected similarly to that of IgG2a, whereas the clearance rate of IgG1 and IgG3 was not affected. The Fc region of IgG2a was required in order for rapid clearance to occur. Biodistribution experiments demonstrated that rapid blood clearance was due, at least partially, to binding to the liver and spleen. To determine the genetic basis for rapid IgG2a clearance, approximately 20 inbred and outbred mouse strains were tested. Unexpectedly, nu/+ as well as nu/nu outbred Swiss mice displayed rapid clearance, whereas control +/+ mice did not, so this phenotype appears to be a dominant effect of the nu mutation. BALB/c nu/nu and nu/+ mice did not display rapid clearance, which may be due to expression of the Igh-1* gene, which codes for the IgG2a present in BALB/c mice and in the monoclonal antibodies used in these studies. In conclusion, this clearance effect must be considered in experiments involving murine IgG2a or IgG2b antibodies in outbred Swiss nude mice, except those in which high antibody doses of >0.1 mg/mouse are used. One method of circumventing this problem is to increase the antibody dose injected; a better but more long-range method is to develop strains of outbred nude mice that do not have this characteristic.

INTRODUCTION

Experiments in immunotherapy and immunodetection often utilize nude mice, partly because human tumor xenografts can be grown in these mice, and partly because the effects of antibodies can be investigated separately from effects of T cell immunity. We report here the unexpected finding that most outbred Swiss nude mice, as well as nu/+ mice, have a very rapid blood clearance rate of murine IgG2a and IgG2b antibodies. Of mouse IgG mAbs, the IgG1 subclass is most common, but IgG2a and IgG2b mAbs are also produced in significant numbers. IgG2a and IgG2b mAbs, but not IgG1s, are active in complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (1, 2), and are therefore of particular importance in many immunotherapy experiments. Accordingly, rapid clearance of IgG2a and IgG2b poses a major obstacle for many experimental systems. We here describe some basic characteristics of this phenomenon, including its relationship to strain of mouse, age, IgG subclass, and dose of Ab.

MATERIALS AND METHODS

Mice and Cell Lines. Outbred nude mice were obtained from either Harlan Sprague-Dawley (Indianapolis, IN), Charles River Laboratories (Wilmingtom, MA), or the Animal Resources facility at Memorial Sloan-Kettering Cancer Center (New York, NY). Outbred normal mouse strains, and the inbred strains BALB/c, C57BL/6, DBA/2, and C3H, were obtained from Harlan Sprague-Dawley. Other inbred strains were obtained from The Jackson Laboratory (Bar Harbor, ME). Except where noted, mice were 6–10-week-old females. In some experiments, nude mice were given injections of 10⁷ cells of the human lymphoma Raji (American Type Culture Collection, Rockville, MD) s.c. after total body irradiation of 350 rad to promote tumor growth, and used for localization studies when tumors reached a size of 0.2–0.5 g.

Monoclonal Antibodies. The mouse antibodies used have been previously described, and include 3 IgG2a mAbs [MA103 (3), TA99 (4), and EPB-2 (5)], 1 IgG2b [MM104 (6)], 1 IgG1 [NP-4 (7)], and 2 IgG3 mAbs [MC56 (8) and FLOPC21, which was obtained from Organon Teknika (Malvern, PA)]. All except FLOPC21 react with antigens on human tumor cells. They were purified by protein A affinity chromatography and labeled with 125I or 111I by the chloramine T method, as described previously (8). By analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, at least 90% of the radiolabel migrated as heavy or light chains of IgG, as shown previously for similar preparations (8). The F(ab’')₂ fragment of EPB-2 was prepared as described (9).

Blood Clearance Rate and Biodistribution. A total of 10⁶ cpm of 0.1–2.0 µg IgG were injected i.v. either in the tail vein or the suborbital venous plexus of the eye (8). A blood sample was collected immediately after injection, at approximately 30–45 s after injection, and later bleedings were obtained at 5, 24, and 72 h, routinely. At each time point, 3–4 drops of blood were collected, after nicking the tail with a scalpel, into preweighed tubes, weighed, and counted (8). We routinely tested 3 individual mice of each inbred strain, 4 individuals of each outbred strain, and 10 individual nu/nu or nu/+ mice of each group, due to the heterogeneity of these mice. In some experiments, varying amounts of unlabeled IgG or F(ab’’₂) fragment of different subclasses were mixed with the labeled IgG before injection. Biodistribution of Abs in tissues was determined as described previously (9). Whole body clearance, in mice given injections of approximately 50 µCi, was measured by 2 methods: with an isotope calibrator (model 34-056, Nuclear Associates) and with a Geiger counter (model E-520, Eberline Instruments, Santa Fe, NM), holding the probe lightly against the ventral surface of the mouse.

RESULTS

Blood Clearance of IgG2a mAbs in Nude Mice. Initial observations were made in nude mice bearing human tumor xenografts, in experiments intended to demonstrate localization of mAbs. We found that the blood level of mAb at 1–14 days after
injection was extremely variable between individual mice, and was often much lower than expected. The distribution of radioactivity in tissues indicated that in mice with very low blood levels the Ab was taken up primarily by the spleen with a lower level of uptake by the liver (Fig. 1). Similar results were obtained with IgG2a mAbs that were either reactive or nonreactive with the transplanted tumors. Such results were obtained with 3 different IgG2a mAbs, but were never observed in a large number of previous experiments using IgG1 mAbs. Further investigation demonstrated that similar results were obtained in non-tumor-bearing nude mice, and that the rapid clearance could be detected at 5 h after injection. Normal BALB/c mice showed the expected 4–5-day T½, when tested with the same Ab preparation (Fig. 2). Fig. 2A shows results with 10 individual nude mice, demonstrating both the rapid clearance in most mice and the marked individual variation. The mice used initially were outbred nude mice from Harlan Sprague-Dawley. To determine whether the source of mice was a significant variable, 10 outbred nude mice were tested from each of 2 other sources, namely Charles River Laboratories and the Animal Resources facility at Memorial Sloan-Kettering Cancer Center. Generally similar results were obtained in all cases. Most experiments utilized female mice, but males were also tested, with very similar results. The mice tested in these experiments were 7–10 weeks of age. To investigate the effect of age on blood clearance rate, mice that had previously displayed a rapid clearance rate were retested at 4 months of age: at this time, all mice had a slow clearance rate, comparable with that in normal BALB/c mice. This change was not due to repeated testing, since younger mice showed rapid clearance in repeated experiments. This change in IgG2a blood clearance rate with aging in individual mice was observed in only a single experiment so we cannot conclude that the change was due to aging itself rather than some environmental effect. However, we can conclude that clearance results in individual nude mice may vary markedly in repeated tests, and such variation may be related to the age of the mice. Since 4-month-old nu/+ mice continued to display rapid IgG2a blood clearance (see below), IgG2a blood clearance is not strictly related to age.

Since variability between individual nude mice is an important aspect of these results, we report in Table 1 a summary of all experiments with nude mice less than 10 weeks old. In 4 other experiments, not listed in Table 1, a total of 20 nude mice was dissected at 3 days after Ab injection; the results were consistent with the data in Table 1, in that more than half of the mice had <1% ID/g blood, whereas other mice had relatively normal levels of 5–10% ID/g blood. In summary, approximately 25% of nude mice had clearance rates that were approximately normal, similar to that in BALB/c mice, whereas 75% had rapid clearance rates. There was some variation between different groups of mice, but at present such variation cannot be attributed to the source, sex, or age of the mice.

Inhibition of Rapid IgG2a Clearance by an Increased Amount of IgG2a. These experiments were performed with nude mice that had been preselected for rapid clearance of IgG2a. The amount of IgG2a routinely injected was 70–250 ng. The effect of increasing the dose, up to 300 µg, was tested. As shown in Fig. 3, 91 µg IgG2a essentially prevented rapid clearance, resulting in a normal T½. A dose of 30 µg had a partial effect, resulting in an intermediate clearance rate. A similar inhibition was observed when TA99 clearance was inhibited by 0.2 mg of a different IgG2a, EPB-2. In contrast, 0.2 mg of the F(ab')2 fragment of EPB-2 or an IgG1 mAb, NP4, did not have a detectable inhibitory effect on the rapid clearance of TA99. In other experiments, the biodistribution of the F(ab')2 fragment of EPB-2 was monitored in nude mice; the clearance rate was very similar to the clearance rate of the F(ab')2 fragment of an IgG1 (9), with a half-life of approximately 10–15 h, and a marked variation between individual mice was not observed. In one experiment, four nude mice were given simultaneous injections of 125I-labeled whole EPB-2 and 131I-labeled F(ab')2 fragment of EPB-2. Two of these mice showed rapid clearance of whole EPB-2, whereas 2 had a normal clearance rate. In contrast, all 4 displayed similar clearance rates for EPB-2 F(ab')2, with 6.5 ± 0.7% (SD) of the injected dose/g of blood present.
clear IgG2a very rapidly, much like nude mice (Fig. 2B; Table 1). Similar results were obtained with CD-1 nu/+ mice from Charles River Laboratories. Variability between individual mice, as observed with nude mice, was seen with the CD-1 nu/+, but not with the Harlan Sprague-Dawley nu/+, for which 26 of 26 mice displayed rapid clearance. The age dependence of the effect noted above with nude mice was not seen with HSD nu/+ mice, since such mice were tested to an age of 4 months, and continued to show rapid IgG2a clearance. We conclude that the nude phenotype is not required to produce rapid IgG2a clearance. Suspecting, at this time, that genes other than nu present in outbred Swiss mice were responsible for the effect, we then tested 10 inbred mouse strains, in an attempt to identify strains that had rapid IgG2a clearance. The strains tested included BALB/c, C57BL/6, DBA/2, C3H, SJL, SWR, AKR, PL/J, BUB, and RE/J, some of which are considered to be derived from outbred Swiss mice (10). All strains tested displayed relatively slow clearance, comparable with that of BALB/c, and none was similar to the outbred nu/+ mice.

We next tested outbred mice, including CD-1, CF-1, ND/4, and ICR; all of these strains had a normal clearance rate of IgG2a. The most informative strain is CD-1, since Charles River Laboratories maintains their outbred nude mice on a CD-1 background, with regular intercrossing to ensure that the background genes do not drift apart. Since CD-1 nu/nu and nu/+ mice displayed rapid clearance, while CD-1 normal mice had normal clearance, we conclude that rapid IgG2a clearance is a dominant effect of the nu gene. The only inbred nude mouse strain included in this study was BALB/c. Both nu/nu and nu/+ BALB/c mice were tested, and both had a normal IgG2a clearance rate. Therefore the nu gene alone is not sufficient to induce rapid IgG2a clearance, but a second genetic factor, absent in BALB/c mice, is also required. A simple explanation, which remains to be investigated, is that normal production of the Igh-1" gene product, which is BALB/c IgG2a, blocks rapid IgG2a clearance, much as clearance in outbred nudes is inhibited by injection of higher doses of IgG2a. The Abs used in these studies were, of course, derived from BALB/c mice. This

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<th>Genotype Source</th>
<th>Age (wks)</th>
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<th>Sex</th>
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* In the top section of Table 1, "Fast" is <1% ID/g blood at 24 h after injection; "Intermediate" is <7% ID/g blood at 24 h after injection. In the bottom sections of Table 1, "Fast" is <5% ID/g blood at 5 h after injection and <3% ID/g blood at 24 h; "Intermediate" is <5% ID/g blood at 24 h after injection.
hypothesis therefore suggests that a non-BALB/c Igh-1 allele may be required to observe rapid clearance.

Site of Uptake of IgG2a and Rate of Excretion. Preliminary experiments indicated that in nude mice with rapid clearance of IgG2a, uptake was primarily in the spleen and liver (Fig. 1). However, dissection in these experiments was performed at 24 h after Ab injection, which is many hours after most of the IgG2a has been cleared from the blood. To investigate earlier events, dissections were performed at 6 h and 1 h after Ab injection into nu/+ mice. At 6 h after IgG2a injection, the blood level ranged from 2–7% (mean 4.7%) of the injected dose/g. To reveal the site of IgG2a uptake most clearly, we selected mice for dissection that had <3.5% ID/g blood at 6 h, which is approximately 4–5-fold lower than the blood level in normal mice at this time. Thus 75–80% of the circulating IgG2a had been removed from the blood in these animals. Results, shown in Fig. 5, demonstrate that much, though not all, of the IgG2a removed from the blood was present in the spleen and liver. The percent ID/g for the spleen was 7.1-fold higher in nu/+ mice than in control CF-1 mice, whereas the percent ID/g for the liver was elevated 3.3-fold. However, since the liver is approximately 4–5-fold lower than the blood level in other tissues examined (kidney, lung, and blood) account for only 3.8 ± 0.8% were present in the liver of the control CF-1 mice. Since the liver and the spleen together accounted for only 15.2% of the injected dose at 6 h after injection, and since the other tissues examined (kidney, lung, and blood) account for only another 7.3%, it is possible that other major sites of IgG2a uptake remain undetected. In the mice dissected at 1 h after Ab injection, although only approximately half of the circulating IgG2a had been cleared from the blood at this time, uptake by the spleen and, less markedly, the liver was clearly observed (data not shown).

A comparison of Fig. 1 with Fig. 5 suggests that the amount of IgG2a bound to the liver and spleen decreases gradually from 6 to 24 h, presumably due to degradation. This was shown directly by monitoring the whole body clearance rate of 125I-EPB-2. Mice were given injections of approximately 50 μCi of 125I conjugated to approximately 5 μg of Ab EPB-2, and whole body radioactivity was determined at various times by 2 methods: a Geiger counter, with the detector placed directly on the ventral surface of the mouse, and a dose calibrator. The Geiger counter was more sensitive at detecting the low level of counts present in nu/+ mice more than 4 days after injection, so cpm obtained with the Geiger count are presented in Fig. 6, but consistent results were obtained with the dose calibrator. In this experiment, blood clearance in the nu/+ mice was considerably slower than in previous experiments, which we tentatively attribute to the relatively high protein dose, 5 μg, injected per mouse. Data in Fig. 3 are consistent with this possibility, since a 30-μg protein dose of IgG2a produced a substantial delay in blood clearance, which however was still much faster than in control mice. Of the 8 nu/+ mice given injections in this experiment, 5 had relatively fast blood clearance rates, with <8.0% of the injected/dose per g of blood at 24 h, and a mean ± SD of 5.1 ± 2.4%. In comparison, the blood level in the control CF-1 mice at 24 h in this experiment was 21.0 ± 1.1% of the injected dose/g, or approximately 4-fold higher. To show clearly whether the IgG2a removed from the blood was rapidly degraded and excreted, whole body counts were obtained for the 5 nu/+ mice that demonstrated rapid blood clearance, and for control CF-1 mice. As shown in Fig. 6, the T1/2 for whole body clearance in nu/+ mice was 31 h, which was approximately 6-fold shorter than the T1/2 in CF-1 mice. Since the T1/2 for excretion was longer than for blood clearance, the rate-limiting step for excretion appears to be the degradation of bound IgG2a.

**DISCUSSION**

We here describe a problem that must be considered in experimental studies of immunotherapy or immunoassay that utilize IgG2a or IgG2b mAbs in outbred Swiss nude mice. The clearance rate of IgG2a was more than 20 times faster than the clearance rate in BALB/c mice, and is sufficiently rapid so that most Ab injected i.v. is cleared and degraded before it reaches interstitial fluid. Since equilibration of intravascular Ab with interstitial fluid requires 12–24 h (11), and since full penetration of solid tumors by intact Ab requires approximately 3 days (12), such rapid clearance severely limits the ability of Abs to reach cells of solid tumors or sites of infection. This rapid clearance can probably be considered an experimental artifact, since it does not occur in BALB/c mice, from which the mAbs available were derived. Still, it must be considered in attempts to use Abs in mice for therapeutic or diagnostic purposes. Previous descriptions of the clearance rate of mouse IgG subclasses in normal mice (2, 13) are consistent with our results in BALB/c mice.

It might be argued that a blood clearance T1/2 of 5 h might be an advantage for some purposes, although it limits the fraction
of the injected Ab that can bind to the tumor. However, the other critical observation is that commercially available populations of outbred Swiss nude mice varied markedly in their IgG2a clearance rate, as exemplified in Fig. 1. In this experiment, the 8 mice tested, which were age- and sex-matched, clearly were divided into 2 groups, of 3 and 5 mice, which differed greatly in their metabolism of IgG2a. In 9 experiments utilizing groups of from 3 to 10 mice, marked variation between individuals was observed in every case except one. Such variation, which was never observed among inbred mice, interferes with the ability to perform interpretable and reproducible experiments.

We have described 4 methods by which rapid clearance in nude mice can be circumvented. First, a relatively high dose of Ab can be utilized, approximately 100 µg per mouse. Second, the use of older mice, at least 4 months of age, probably would reduce the incidence of rapid IgG2a clearance. However, this suggestion is based on results with a single group of 4 nude mice, who displayed rapid IgG2a clearance when 2 months old and normal clearance when older, and this age effect was not observed with nu/+ mice. Thus, further validation of this approach is clearly required. Both of these approaches must be used cautiously, due to the fact that there is great variation between individual nude mice. Hence, it seems essential to monitor blood clearance of individual mice to ensure that the Ab remains in circulation long enough to produce the intended effect. A useful guideline would be that at least 10% ID/g blood should be present at 1 day after injection, and at least 5% at 3 days after injection, if the blood clearance rate is normal.

The third solution to this problem is to use BALB/c nude mice, which do not show rapid blood clearance of IgG2a. However, BALB/c nude mice are available only in limited quantities and are quite expensive, and therefore cannot readily replace outbred nude mice. A better solution, though more long-term, is to generate outbred nude mice that do not show rapid IgG2a clearance. If the IgH-1* allele, or some other single gene expressed in BALB/c mice, is sufficient to inhibit rapid clearance, as we hypothesize, then this gene can be inserted into an outbred Swiss background. The fourth approach is to use the F(ab')2 fragment of mAbs, since these are not affected by the rapid clearance mechanism described; however, this does not entirely resolve the problem, since F(ab')2 fragments themselves have a relatively rapid blood clearance rate (9).

The expression of rapid IgG2a clearance in nu/+ mice is notable, since the nu gene has generally been considered recessive (14). Holub (15) did report that nu/+ mice were slightly different from +/+ animals, and tended to resemble nu/nu control mice, but such variation was relatively minor, in comparison to the striking dominant effect observed here. Fechner et al. (16) reported several immunological differences between nu/+ and +/+ BALB/c mice, but their results may have been due to the misidentification of outbred nude strains derived from the NIH as BALB/c, a problem that was recognized in 1982.* Nu/+ mice therefore do not always constitute a suitable normal control population, particularly if the experiment involves injection of BALB/c IgG2a or IgG2b Abs. The mechanism by which the nu gene induces rapid IgG2a clearance requires further investigation. One intriguing possibility is that the nu gene product is expressed and acts directly as a receptor for BALB/c IgG2a, or modifies the function of such a receptor. The fact that IgG2a uptake was via both the liver and the spleen suggests that it is distinct from the normal clearance mechanism of IgG, which is evidently primarily via the liver (17). Considering that nude mice have low blood levels of IgG2a, which is partially thymus-dependent (18, 19), we initially considered the possibility that this fact might be related to the rapid blood clearance of IgG2a. However, this is not consistent with the expression of rapid IgG2a clearance in nu/+ mice, which have normal thymus-dependent immunity and normal blood levels of IgG2a (18, 19).

Considering the unexpected nature of the results reported here, it is particularly important to consider other possible explanations. The results cannot be attributed to degraded or denatured IgG preparations, since the same preparation was often tested, at the same time, in groups of mice showing both fast and normal clearance rates. In particular, nu/+ mice having rapid clearance were frequently tested at the same time as inbred or outbred mouse strains having normal clearance. Alterations resulting from iodination do not appear to be involved, since, in the inhibition experiments (Fig. 2), clearance of iodinated IgG2a was blocked by unlabeled Ab.

Nu/+ mice are raised in a nude mouse isolator colony, and therefore in a different environment from conventional mice. However, we have maintained nu/+ mice for up to 3.5 months in conventional mouse rooms, and they continue to display rapid IgG2a clearance. Therefore, it is unlikely that the difference between nu/+ and +/+ is due to environmental effects. This possibility can be definitively investigated by testing nu/+ and +/+ littermates, derived from appropriate matings. However, this experiment and others are complicated by the need to use outbred mice, and by the fact that marked variation between individuals was found for both nu/nu and nu/+ groups. Further analysis would be greatly facilitated by identifying inbred nude mice having rapid clearance, and by identifying the genes present in BALB/c nude mice that prevent rapid clearance.

Since IgG2a mAbs are fairly common, and since radiolocalization experiments in nude mice bearing human tumor xenografts are widely used, it seems surprising that the effects described here were not reported earlier. This may be attributable to the variation between individual mice that we describe here. The basis for such variation, whether genetic or environmental, is not known, but it seems likely that different colonies of outbred nude mice might differ significantly in this regard. In addition, very low blood levels at the time of dissection may sometimes be falsely attributed to a poor injection, and therefore such mice may be omitted from published data; this can be distinguished only if a 0-h blood measurement is obtained. Low blood levels might also be falsely attributed to formation and rapid clearance of immune complexes with antigen released from tumor cells, although this could not affect clearance of control, nonreactive Abs. Therefore, we suggest that rapid blood clearance of IgG2a in nude mice could have been overlooked in previous experiments, and may in fact explain some poor localization results. In immunotherapy experiments, and in our previous experiments with biotinylated Ab (12), large amounts of Ab were injected, which would be expected to block rapid clearance.

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Rapid Blood Clearance of Immunoglobulin G2a and Immunoglobulin G2b in Nude Mice


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