Cancer Immunol Immunother (2002) 51: 547–556
DOI 10.1007/s00262-002-0319-x

ORIGINAL ARTICLE

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Rapid blood clearance of injected mouse IgG2a in SCID mice

Received: 2 April 2002 / Accepted: 8 July 2002 / Published online: 19 September 2002

Abstract SCID mice were found to have rapid blood clearance of injected mouse IgG2a antibodies (Ab), while IgG1 Ab were cleared normally. This effect varied depending on the strain of SCID mice, being very rapid in most Taconic ICR mice and slower in C.B-17 mice. A similar effect was previously described in nude mice, and shown to be due to the very low concentrations of endogenous IgG2a and IgG2b in these mice. Therefore, IgG2a and IgG2b concentrations were assayed in sera from 30 SCID mice: the concentrations were very low, with one exception. Rapid blood clearance in all strains could be strongly inhibited by injection of large amounts of irrelevant IgG2a. Therefore, the low endogenous IgG2a and IgG2b concentrations are responsible for the rapid blood clearance rate of injected IgG2a in these mice, as in nude mice. However, the relatively slow blood clearance of injected IgG2a in certain SCID mice (C.B-17 mice and rare Taconic ICR mice), was not generally due to higher levels of IgG2a or IgG2b, but rather to some other factor that has not been identified. $F(ab')_2$ Ab fragments were not cleared rapidly, which supports other evidence that clearance is via the CD64 Fcγ receptor. This rapid clearance of IgG2a affects the ability of such Ab to target tumors, and was also shown to affect the maximum tolerated dose (MTD) of radiolabeled IgG2a Ab, labeled with either ^{125}I or ^{111}In. Mice with faster blood clearance had a lower MTD, probably due to the fact that Ab uptake was in the bone as well as the spleen. This effect must be taken into consideration in experiments in which SCID mice are injected with IgG2a or IgG2b Ab.

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Keywords Antibody immunotherapy Fc receptor · IgG2a blood clearance · SCID mice

Introduction

As antibody (Ab) therapy has become established as an effective method of cancer therapy, it is important to further develop useful experimental models. We and others have previously described an obstacle to the use of nude mouse xenograft models, namely the very rapid blood clearance of injected mouse IgG2a or humanized IgG1 Ab [18, 25]. This clearance is to the spleen and liver, and it is sufficiently fast that it prevents Ab from effectively reaching the intended target, which is a subcutaneous (s.c.) tumor. There is strong circumstantial evidence that this clearance is due to the high-affinity Fc receptor CD64 [18], although direct evidence for the role of this receptor has not yet been found. The effect is a consequence of very low levels of endogenous IgG2a and IgG2b in these mice, since in normal mice the receptors are largely occupied by reversibly bound IgG2a and/or IgG2b that is normally present in the circulation. Since the effect occurred in normal *nu*/+ as well as mutant *nu*/*nu* mice [18], it was evident that the low IgG2a and IgG2b levels in blood were not due so much to the immunodeficiency of the mice (although this may play some role), but primarily to the very clean, pathogen-free environment in which these mice were raised, which results in an immune system that is barely stimulated. While mouse IgG2a Abs were cleared most rapidly, mouse IgG2b Ab were also affected, as were human IgG1 Ab, which is the homologous subclass in humans, and these results are consistent with the specificity of mouse CD64 [4, 17].

Given these results, it would be expected that the same effect would occur in SCID mice, considering that IgG levels in SCID mice are expected to be much lower than those in nude mice [2]. Occasional SCID mice produce high levels of IgG, but this is rare, and occurs mainly in older mice [1]. In this report, we show that rapid IgG2a

clearance does occur, and that it affects the maximum tolerated dose (MTD) that can be used in therapy experiments with radiolabeled Ab. We also investigated the organs responsible for rapid blood clearance. Since this clearance interferes with Ab therapy experiments and with other studies using Ab, we also evaluated methods by which it can be prevented. This can be achieved by injecting sufficient amounts of irrelevant IgG2a, but large amounts are required, approximately 0.25 mg/mouse for the maximum effect. As a practical technique, we have used 0.1 ml of normal outbred CF-1 serum, a source of normal mouse IgG2a, as a competitive blocking reagent. This approach was quite effective, but it does not entirely eliminate the problem, as is discussed.

Materials and methods

Mice, antibodies, and radiolabeling

ICR and C.B-17 SCID mice, as well as normal ICR mice, were purchased from Taconic Labs (Germantown, N.Y.). SCID mice were also purchased from Animal Resources at the National Cancer Institute (Frederick, Md.); these mice were also C.B-17, but herein are referred to as NCI mice. CF-1 mice as well as some pools of CF-1 mouse sera were purchased from Charles River Laboratories (Wilmington, Mass.). The Ab used include three IgG2a Ab (LL2, 1F5 and RG11) and an IgG1 (LL1) that have been described previously, together with the method of purification [6, 7]. LL1 and LL2 were provided by Immunomedics Inc. (Morris Plains, N.J.). An IgG2b Ab, MOPC 195, was purchased from Sigma Chemicals (St. Louis, Mo.). IgG2a and IgG2b Ab of the C57BL/6 allotype were purchased from BD Biosciences (San Diego, Calif.; 03311D and 03301D, respectively). The $F(ab')_2$ fragment of LL2 was prepared by pepsin digestion as previously described [19]. Iodination with ^{125}I or ^{131}I was by standard methods that have been described [7], to specific activities of 10–20 mCi/mg. Ab were labeled with ^{111}In via the chelator benzyl-diethylenetriamine pentaacetic acid (DTPA) as described [7], to specific activities of approximately 10 mCi/mg.

Determination of blood clearance rate and biodistribution

These methods have been described previously in detail [18]. Radiolabeled Abs (generally 10^6 cpm, on approximately 30–60 ng Ab) were injected i.v. into the tail vein in a volume of 0.2 ml, and bleeding was performed from the suborbital plexus. Approximately 50–70 mg of blood was collected at each bleeding, which was carried out routinely at 0.5, 24, and 48 h, and sometimes later. In some experiments, we attempted to distinguish between bone and bone marrow as follows. One femur was weighed and counted for radioactivity normally. The second femur had both ends cut off, and the marrow was flushed out by injecting 0.5 ml of 0.15 M NaCl into both ends. The cpm of the collected marrow and the washed bone (including the ends) was determined, and used to calculate the percentage of the total that was present in the marrow. In double-label experiments, ^{125}I cpm were corrected for cross-over from ^{131}I.

Assay of IgG2a or IgG2b concentrations

The sandwich ELISA for IgG2a has previously been described in detail [18]. The standard Ab (LL2 or 1F5) was used starting at 600 ng/ml, and 5 serial 3-fold dilutions were included. SCID sera were tested at a starting dilution of 1:100. Other sera, with higher concentrations of IgG2a, were tested starting at 1:1,000 or 1:10,000. For all sera, 2 serial 4-fold dilutions were also tested. The assay for IgG2b was performed identically, except that the Ab initially bound to the plate was anti-IgG2b rather than anti-IgG2a (GAM/IgG2b/7S; Nordic Labs, San Capistrano, Calif.). The standard Ab was MOPC 195, used at the same concentrations as described for LL2. Control experiments showed that there was no cross-reaction between IgG2a, IgG2b, or IgG1 in either of the assays. A BALB/c reference serum was included in all assays to check the reproducibility of the results.

Statistics

All comparisons utilized Student's *t*-test, except as otherwise noted.

Results

Blood clearance of IgG2a Abs in SCID mice

Three strains or sub-strains of SCID mice were tested, in part because of substantial differences between strains of nude mice that were previously described. As shown in Fig. 1, there were major differences between the strains

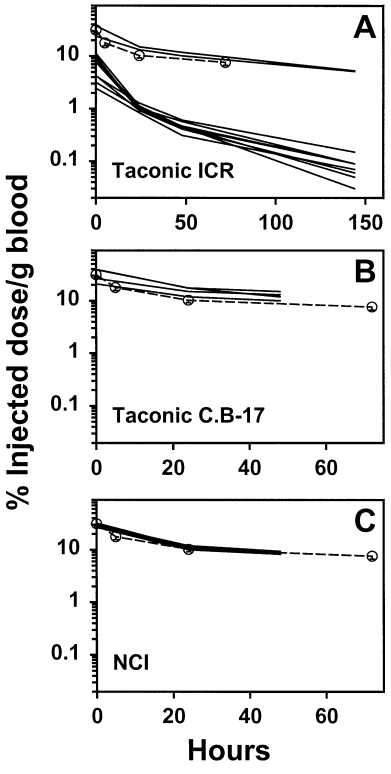

Fig. 1. A–C Blood clearance of injected IgG2a Ab in SCID mice. Each *solid line* represents data from one mouse. **A** Taconic ICR mice injected with ^{131}I-LL2. Five males and 5 females were tested, but they are not distinguished because the results were the same. One male and one female showed normal blood clearance rates, and the other 8 mice showed rapid blood clearance. **B** Taconic C.B-17 mice injected with ^{125}I-LL2; 4 females were tested. **C** NCI mice, injected with ^{125}I-RG11; 5 females were tested (*the lines are partially superimposed*). For comparison, results with BALB/c mice, injected with ^{125}I-LL2, are also shown (*dashed lines with circles*); values shown are means and standard deviations for 3 mice (but all of the standard deviation bars are smaller than the symbols). All mice were bled at 0.5, 24 and 48 h, and some were also bled at 144 h

of SCID mice tested. Fig. 1A shows results with Taconic ICR (outbred) mice. Of the 10 mice included in this figure, 8 showed very rapid blood clearance, and 2 showed normal clearance. Such marked variation between individual mice was characteristic of similar experiments performed with nude mice [18, 21]. Four of the mice with rapid clearance of IgG2a were subsequently (approximately 2 weeks later) tested for clearance of an IgG1 Ab: all had normal, slow clearance of the IgG1, with the percentage injected dose/g of blood in the range of 18.0%to 20.3% at 48 h. In other experiments, 5 additional mice of this strain were tested, and all showed rapid blood clearance. In contrast, the other 2 SCID mouse strains tested displayed normal or nearly normal clearance of IgG2a Ab. Fig. 1B shows the results with Taconic C.B-17 SCID mice, and Fig. 1C shows the results with SCID mice from NCI Animal Resources, which are also C.B-17. Different IgG2a Ab were used in some of the experiments, for convenience, but the effects were independent of the particular IgG2a used. For example, labeled RG11 (used in Fig. 1C), was also injected into Taconic ICR mice and was cleared rapidly, like labeled LL2.

To further confirm the specificity of uptake, experiments were performed with an $F(ab')_2$ fragment of an IgG2a. As shown in Fig. 2, the intact IgG2a was cleared from the blood much faster than the $F(ab')_2$ fragment. Since such fragments are generally cleared much faster than intact Ab, in normal mice, this result demonstrates that rapid clearance requires the Fc region of the Ab, and is therefore likely to be via an Fc receptor, as postulated previously to explain results in nude mice [18].

Levels of endogenous IgG2a and IgG2b in SCID mice

We next attempted to explain the marked difference between the mice in their IgG2a clearance rates. Since in nude mice, in previous studies, the IgG2a clearance rate in individual mice was strongly correlated with the level of endogenous IgG2a in the serum, we assayed the IgG2a concentration in sera from the same mice used in the preceding experiments, with the serum being collected shortly after the blood clearance experiments had been completed. An ELISA to measure IgG2a in the serum was performed. Twenty-five serum samples from individual SCID mice were tested, including all of the mice used in Fig. 1. All IgG2a levels were barely detectable: most were < 0.1 μg/ml, and the 3 highest values were 0.14 μg/ml, 0.25 μg/ml, and 0.7 μg/ml. Sera from normal mice of various strains were tested to confirm the reliability of the assay. These normal sera had IgG2a concentrations ranging from 54 μg/ml to 1,128 μg/ml, which were consistent with the values obtained in previous studies [18]. Based on previous data with nude mice, these IgG2a concentrations are much too low to block rapid clearance of injected IgG2a. Therefore, the slow clearance of IgG2a in some of the SCID mice was not due to endogenous IgG2a. One possible explanation was that IgG2b rather than IgG2a was responsible. IgG2b is known to bind to the CD64 Fcγ receptor, although less avidly than IgG2a [17, 24]. Therefore, an ELISA for mouse IgG2b was employed. Again, the 25 SCID mouse sera tested had very low levels of IgG2b: most were < 0.1 μg/ml, except for one that was 0.15 μg/ml. We conclude that endogenous IgG2b is also not responsible for blocking IgG2a blood clearance in some of the SCID mice.

Since there are multiple allotypes of IgG2a and IgG2b, and since the differences between allotypes is particularly great for IgG2a [13, 28], it was essential to ensure that the IgG2a and IgG2b of certain allotypes reacted well in our ELISA, which used BALB/c Ab as standards. Since the C.B-17 SCID mice have the C57BL Ig heavy chain (*Igh*) allotype, IgG2a and IgG2b of this allotype were tested. The C57BL/6 IgG2a produced exactly the expected reading in the ELISA, meaning that it reacted essentially the same as the BALB/c standard protein. The C57BL/6 IgG2b, however, reacted differently from the BALB/c standard protein, by a factor of 3.0. This difference is significant, but it is not unexpected, given the differences between alleles [13], and it does not affect the conclusions of this study, although it does suggest that IgG2b concentrations in the C.B-17 SCID mice (which were barely detectable, in any case) could have been underestimated by a factor of 3. While the *Igh* allotype of the outbred ICR mice is not known (and there could be more than one), we tested sera from 10 individual normal ICR mice as a control. These sera, from 12-week old males, had a mean IgG2a concentration of 47 μg/ml (range: 10–116 μg/ml), and a mean IgG2b concentration of 48 μg/ml (range: 14–87 μg/ml). Therefore, we conclude that if the ICR SCID mice had substantial levels of IgG2a or IgG2b in their sera, it would have been detected in our assays. These concentrations may appear low, but they are similar to values found in many strains of mice, including BALB/c itself [18]; we have suggested that this is due to the very clean, pathogen-free environment in which mice are raised [18].

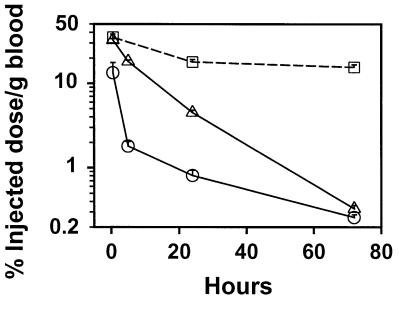

Fig. 2. The Fc region of IgG2a is required for rapid blood clearance. Groups of 5 Taconic ICR SCID mice were injected with 5 μCi of either intact IgG2a (LL2; *open circles*) or the $F(ab')_2$ fragment of LL2 (*open triangles*), labeled with ^{125}I. Values shown are means and standard deviations. Results with an IgG1 Ab in the same mouse strain, from a previous experiment, are also shown, for comparison (*open squares*). Most of the standard deviations are smaller than the symbols

Blood clearance and tissue distribution of IgG2a compared to IgG1 in NCI SCID mice

Although blood clearance of IgG2a was not very rapid in NCI SCID mice, as it was in Taconic ICR SCID mice, we realized that the clearance rate was significantly faster than that of an IgG1 Ab. This is demonstrated in Fig. 3A. Of the 5 mice tested, one was exceptional in displaying IgG2a clearance as slow as the clearance of IgG1. In an attempt to explain the results with this mouse, these 5 SCID sera were also tested for their IgG2a and IgG2b concentrations. Four of the mice had the usual very low concentrations of IgG2a and IgG2b, but the one exceptional mouse (having slow blood clearance of injected IgG2a) had 452 μg/ml of IgG2a and 165 μg/ml of IgG2b. Thus, it appears likely that the slow clearance of IgG2a in this mouse was due to the high level of endogenous IgG2a and IgG2b, although no definite conclusions can be reached on the basis of one animal. Of the 30 SCID mice that were tested for their endogenous serum concentration of IgG2a and IgG2b in this study, this was the only mouse with high levels. High levels of IgG in rare SCID mice have been described previously [1]. To compare the total blood residence time of IgG1 and IgG2a, the area-under-the-curve (AUC) was calculated for the experiment shown in Fig. 3A. For this purpose, mean values were used, and the exceptional mouse (having slow clearance of IgG2a) was excluded. Over the 6-day period, the AUC was 52% higher with IgG1 than with IgG2a. This difference, while much less dramatic than in the ICR SCID mice, is sufficient to have an impact on Ab localization and therapy experiments, and was therefore investigated further.

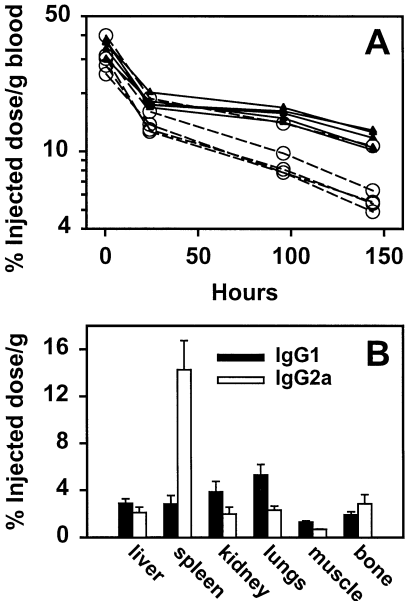

Fig. 3. A–B Difference in blood clearance and tissue distribution between IgG1 and IgG2a in NCI SCID mice. Five animals were injected i.v. with ^{125}I-IgG1 (LL1), and ^{131}I-IgG2a (LL2). **A** Blood clearance of individual mice for IgG1 (*filled triangles with solid lines*) or IgG2a (*open circles with dashed lines*). Note that in one of the 5 mice the IgG2a was cleared slowly, but in the other 4 mice the IgG2a was cleared substantially faster than the IgG1. **B** Tissue distribution of the radiolabels in the same mice, dissected at the time of the last bleeding (144 h). Means and standard deviations are shown. The exceptional mouse having slow clearance of IgG2a was omitted from the calculations; this mouse had tissue distribution of IgG2a very similar to that of IgG1, with the spleen level of IgG2a being 2.8%. Note the high level of IgG2a uptake in the spleen, and a lower level of uptake in the bone

To analyze the IgG2a clearance mechanism, the mice used in Fig. 3A were dissected at the time of the last bleeding. Fig. 3B shows the percentage injected dose/g of tissue. Again, the exceptional mouse having high endogenous IgG concentrations and slow clearance of IgG2a was omitted from the calculations. The difference in uptake between IgG2a and IgG1 was statistically significant for all tissues examined ($P < 0.05$). Uptake was most prominent in the spleen, as expected [21], with $P < 0.001$. Most tissues (kidney, lungs, and muscle) had a lower level of IgG2a than of IgG1, which can be attributed to the lower blood level, and indicates that the IgG content of these tissues is primarily due to their blood content. The liver also had a lower level of IgG2a than of IgG1, but the difference was less than with the other tissues mentioned, suggesting that there was a low level of IgG2a uptake by the liver, which would be consistent with previous results [21], and can be attributed to Kupffer cells in the liver, which express CD64 [8]. Thus, the ratio of (% injected dose/g for IgG2a)/(% injected dose/g for IgG1) was 5.04 for the spleen, and 0.72, 0.51, 0.43, 0.46, and 0.52 for the liver, kidney, lungs, blood, and muscle, respectively. This ratio was 1.49 for bone, and Fig. 3B also shows substantial IgG2a uptake by bone. This uptake in bone is probably due to macrophage-lineage cells in the bone marrow, which express CD64 [8], and also to CD64 expression on immature myeloid cells [8]. Bone was not investigated in the previous experiments with nude mice.

An attempt was made to determine if the radioactivity in the bone was present in the bone marrow or in the bone itself. The second femur (not used in the biodistribution analysis) was flushed with saline to remove the marrow, and both the marrow and the residual bone were counted for radioactivity. This experiment provided some indication that there was uptake of IgG2a in the marrow, but the results were too variable to be conclusive, probably because of the technical difficulty of expelling marrow from the bone. The percentage of IgG1 in the marrow was $14.3 \pm 6.7\%$, and the percentage of IgG2a in the marrow was $22.6 \pm 8.9\%$ ($P < 0.2$).

Tissue uptake of radioiodine can be underestimated if the labeled protein is degraded rapidly, since catabolites rapidly leave the cell. This is prevented by using "residualizing" labels such as ^{111}In-benzyl-DTPA, for which catabolites are trapped in lysosomes [22]. Therefore, similar experiments were performed with ^{111}In labels. Groups of mice were injected with either ^{111}In-IgG1 or ^{111}In-IgG2a. This experiment was intended not only to reveal more clearly the sites of uptake of IgG2a, but also to show whether catabolism is

fast after uptake. Results, shown in Fig. 4A, are generally similar to those with iodine labels, but there appears to be somewhat more accumulation of the ^{111}In, especially in the spleen, liver, and bone marrow. The difference in uptake between IgG2a and IgG1 was statistically significant ($P < 0.05$) for all tissues examined except the liver and muscle. The ratio of (% injected dose/g for IgG2a)/(% injected dose/g for IgG1) was 1.04 for the liver, 6.72 for the spleen, 0.72 for the kidney, 0.64 for the lungs, 0.45 for the blood, 0.79 for the muscle, and 2.69 for the bone. Thus, we now see some accumulation of IgG2a in the liver: although the amount appears to be small, this accumulation must compensate for the much lower blood level of the IgG2a (since much of the Ab present in the liver is due to its blood content), so in fact the accumulation in the liver is clear. These data confirm the previous conclusions from the iodine experiments. Distinction between bone and bone marrow was also attempted in this experiment. The percentage of IgG1 in the marrow was $14.9 \pm 3.6\%$ and the percentage of IgG2a in the marrow was $20.2 \pm 12.1\%$, so very similar to results with the iodine label. Thus, there was again a tendency for greater uptake of the IgG2a, but the difference was not significant ($P > 0.2$), due to variability between mice.

Fig. 4B shows a comparison between iodine and indium labels on IgG2a, using the data from Figs. 2 and 3A. If there is greater uptake of indium than iodine, this is an indication of catabolism in that tissue. The blood level was the same in both experiments, as it should be. There was significantly higher uptake of indium in all of the other tissues ($P < 0.01$ for all 6 tissues), but the difference was greatest for the liver, spleen and bone. The ratio of (% injected dose/g for ^{111}In)/(% injected dose/g for ^{125}I) was 2.53 for the liver, 1.93 for the spleen, 1.59 for the kidney, 1.61 for the lungs, 1.03 for the blood, 1.68 for the muscle, and 2.24 for the bone marrow. Thus, there is some indication that catabolism is faster in the liver and bone marrow than in the spleen. The fact that IgG is normally catabolized in all normal tissues, with the liver and spleen being most active relative to their mass, has been previously established [9]. We conclude that the difference in distribution between IgG1 and IgG2a, while seen clearly with both iodine and indium labels, is somewhat greater with the residualizing indium label, due to some catabolism after uptake.

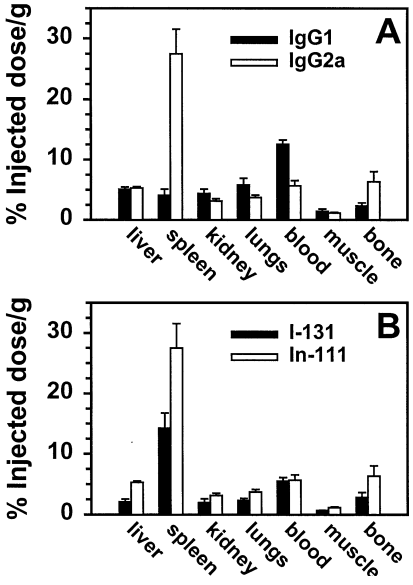

Fig. 4. **A** Differences in tissue distribution between ^{111}In-labeled IgG1 (LL1) and IgG2a (LL2) in NCI SCID mice. Groups of 5 animals were injected i.v. with labeled Ab, and dissected at 144 h. Means and standard deviations are shown. **B** A comparison of the tissue distribution of IgG2a labeled with either ^{131}I or ^{111}In. Data are from Fig. 3 and Fig. 4A

Differences between IgG1 and IgG2a in the MTD of radiolabeled Ab

This difference in clearance between IgG1 and IgG2a will affect Ab uptake in an s.c. tumor, and also is likely to have some effect on the non-specific toxicity of radiolabeled Ab. But it is not obvious a priori whether faster blood clearance would result in a higher or lower MTD. (We define the MTD as the maximum dose at which all mice survive.) It is interesting to note, for example, that in our radioimmunotherapy (RAIT) experiments, we routinely use the calculated radiation dose to the blood as an indicator of bone marrow dose, and the correlation has been found to be useful in a wide range of experiments [14, 20]. Under most circumstances, bone marrow toxicity is the dose limiting toxicity in RAIT. If this factor alone were considered, then faster blood clearance should result in a lower bone marrow dose. But it is evident that this will depend on the particular normal organs that are involved in the clearance, and uptake in bone or bone marrow may cause a reduction in the MTD. Results of MTD experiments in NCI SCID mice with ^{111}In-labeled Ab are summarized in Fig. 5. The MTD was significantly higher with an IgG1 Ab, i.e. 300 μCi, than with an IgG2a Ab, i.e. 160 μCi. It seems reasonable to suggest that the lower MTD for IgG2a is due to bone uptake. We note that ^{111}In is generally not considered to be a therapeutic radionuclide, but we recently demonstrated that emitters of low energy electrons, such as ^{111}In and ^{125}I, conjugated to Ab, can provide effective therapy in a mouse xenograft model [11], and can also kill cells in vitro efficiently and specifically [12]. Fig. 5 also includes data from experiments in which normal mouse IgG2a was used to block rapid blood clearance, which are discussed below.

Inhibition of rapid clearance of IgG2a by co-injection of normal mouse IgG2a and effect on the MTD

It seemed that it might be possible and useful to delay the clearance of IgG2a in NCI SCID mice by injection of

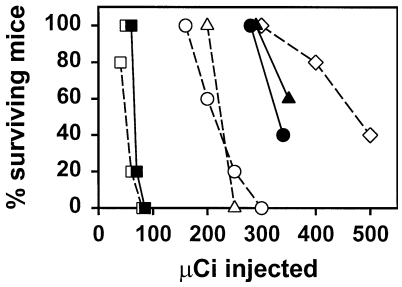

Fig. 5. Determination of the MTD of various radiolabeled Ab in NCI SCID mice. Injected IgG2a (LL2) was: labeled with ^{111}In and injected without (*open circles*) or with (*filled circles*) CF-1 serum; labeled with ^{131}I and injected without (*open squares*) or with (*filled squares*) CF-1 serum; labeled with ^{125}I and injected without (*open triangles*) or with (*filled triangles*) CF-1 serum. The IgG2a concentration in the CF-1 serum was 1.86 mg/ml for the iodine experiments and 807 μg/ml for the ^{111}In experiment. Results with ^{111}In-IgG1 (LL1) are also shown (*open diamonds*). Groups of 5 mice were used. The results for ^{131}I were from 2 experiments, which is why all of the data points are not connected

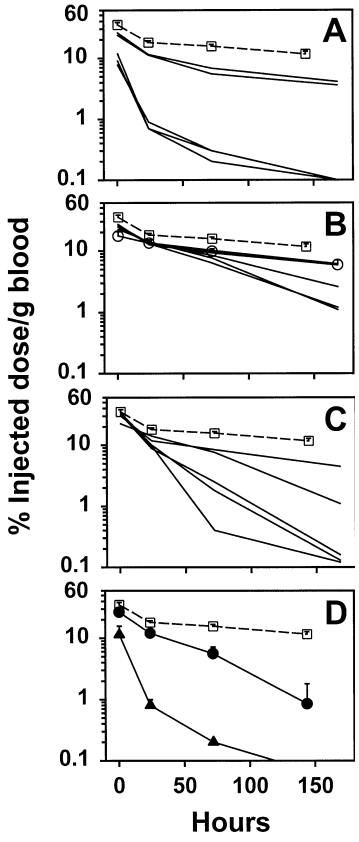

Fig. 6. A–D Effect of co-injection of irrelevant mouse IgG2a on blood clearance of ^{125}I-labeled IgG2a (1F5) in Taconic ICR SCID mice. Mice were injected with 5 μCi. For comparison, the blood clearance rate of an IgG1 in mice of the same strain is also included in all 4 panels (*open squares*). **A** Blood clearance in 5 control mice, injected with the radiolabeled Ab only, showing rapid clearance in 3 of the 5 mice. **B** The effect of co-injection of purified irrelevant IgG2a (LL2). For 5 mice injected with 250 μg, the means and standard deviations are shown, since all 5 mice had similar rates of blood clearance (*open circles*). For 5 mice injected with 83 μg, the results of individual mice are shown, since there was significant variation between the mice, especially at the last time point. Two of these lines are nearly superimposed on the line obtained with the 250 μg dose. **C** Results with 5 individual mice injected with 28 μg are shown. **D** The effect of co-injection of 0.1 ml normal CF-1 serum (*filled circles*). This experiment was performed with a different batch of mice, so another control group of 5 mice was included (*filled triangles*); these 5 mice all displayed rapid IgG2a blood clearance. Means and standard deviations are shown. The IgG2a concentration in this batch of CF-1 serum was 845 μg/ml. The effect of CF-1 serum is statistically significant ($P < 0.001$) at 0.5, 24 and 48 h. (Many of the standard deviation bars are smaller than the symbols)

normal mouse IgG2a, which would compete for binding to CD64. Previous results with nude mice [18], as well as the results described above with the exceptional SCID mouse having high levels of endogenous IgG2a and IgG2b, suggested that this would be effective. To investigate this possibility, mice were co-injected with ^{125}I-IgG2a and varying amounts of a different, unlabeled IgG2a, using doses ranging from 28 to 250 μg/mouse. Taconic ICR SCID mice were used for this experiment, since they generally display faster clearance of IgG2a than the other SCID mice that have been tested. As shown in Fig. 6B, 250 μg IgG2a provided strong inhibition of clearance over a period of 7 days; 83 μg produced effective inhibition for 3 days, but by day 7 the inhibition was beginning to wane in 3 of the 5 mice. A dose of 28 μg (Fig. 6C) produced much less inhibition, with effective inhibition for only 1 day, and marked heterogeneity between the individual mice. Results with the 5 control mice used in this experiment, shown in Fig. 6A, also demonstrate marked heterogeneity between mice, with 2 distinct populations being present: 3 of the 5 mice showed rapid clearance, which the other 2 showed slow clearance. Such variation between individual mice is characteristic of this phenomenon [18, 21], and was also observed in Fig. 1, in which 8 of 10 Taconic ICR SCID mice had rapid clearance, while the other 2 had slow clearance. We note that the mice used in Fig. 2 were from the same batch of mice as used in Fig. 6A, and were used only 1 week later; all 5 of those mice displayed rapid clearance of IgG2a. Thus, of the 10 mice in this batch that were tested for clearance of IgG2a, 8 had rapid clearance and 2 had slow clearance. This variation must be considered in interpreting the results shown. For example, in Fig. 6C, the fact that 2 of the mice showed slow blood clearance may not be due to the effect of the inhibitory IgG2a injected, but rather to the fact that these 2 mice are among the group that would normally display a slow clearance rate. Despite this variation, the inhibitory effects of irrelevant mouse IgG2a were clear. While 250 μg was required for near-complete inhibition over 7 days, a dose of 83 μg would be satisfactory for many purposes, since clearance was blocked for at least 3 days.

Since there is no need for purified IgG2a for the purpose of inhibition, and since use of large amounts of purified IgG2a, which would be required, is expensive, we attempted to use normal CF-1 mouse serum as a convenient and inexpensive source of normal mouse

IgG2a. A previous survey of IgG2a levels in sera from mice of various strains suggested that this strain had relatively high levels [18], although it should be noted that only a few animals of each strain were tested. A batch of CF-1 serum was collected from 7-month-old females, and had 845 μg/ml IgG2a, according to the ELISA. Fig. 6D shows the effect of co-injection of 0.1 ml of normal CF-1 serum on IgG2a clearance. This resulted in a substantial decrease in the clearance rate, but clearance was still considerably faster than the clearance rate of an IgG1. By comparison with the studies using purified IgG2a, the inhibitory effect of normal CF-1 serum was just as expected based on its IgG2a content (with 85 μg present in the 100 μl injected), and we can therefore conclude that the effect is probably due to the IgG2a, and not to other components of the serum. The AUC with IgG2a plus CF-1 serum was 6.3-fold higher than it was in control mice without CF-1 serum, but was still 54% lower than with an IgG1. We conclude that, in these mice, co-injection of CF-1 serum provides effective inhibition of clearance for approximately 3 days, which is long enough for many experimental purposes, but is less effective for later time points.

In NCI SCID mice, since the rapid clearance of IgG2a is much less dramatic, it might be expected that inhibition with irrelevant IgG2a might be more effective. In fact, co-injection of CF-1 serum, together with radiolabeled IgG2a, resulted in a clearance rate that was now similar to that of IgG1 (Fig. 7). The effect of CF-1 serum was statistically significant at 24 h ($P < 0.02$) and at later time points ($P < 0.01$). These mice were also dissected at the conclusion of the experiment (Fig. 7B): the splenic uptake was significantly reduced due to the co-injection of CF-1 serum ($P < 0.05$). Liver uptake decreased only slightly, but this is an indication of lower specific uptake in the liver, since the much higher blood levels of Ab, in itself, would cause higher uptake in all organs, as is the case for the kidney, lungs, and muscle. Bone was not included in this experiment.

To demonstrate that the decreased blood clearance rate produced by co-injected CF-1 serum could increase the MTD, studies were performed with ^{111}In, ^{125}I, and ^{131}I-labeled IgG2a. Results are included in Fig. 5. Co-injection of CF-1 serum caused a substantial increase in the MTD. For ^{111}In, the increase was from 160 μCi to 280 μCi; for ^{125}I, the increase was from 200 μCi to 290 μCi; for ^{131}I, there appeared to be a slight increase from 35–50 μCi to 60 μCi. (The MTD with ^{131}I-IgG2a is uncertain because in the first experiment 40 μCi killed 1 of 5 mice, while in the second experiment all mice survived 50 μCi.) It appears therefore that this difference is greater for the Auger electron emitters (^{111}In and ^{125}I) than for the β-particle emitter (^{131}I). Since batches of mice vary somewhat in age, size, and other characteristics, the difference in MTD was confirmed in a separate experiment. Groups of 5 mice were injected with 280 μCi ^{111}In-LL2, with or without 0.1 ml CF-1 serum. All mice co-injected with CF-1 serum survived, and remained

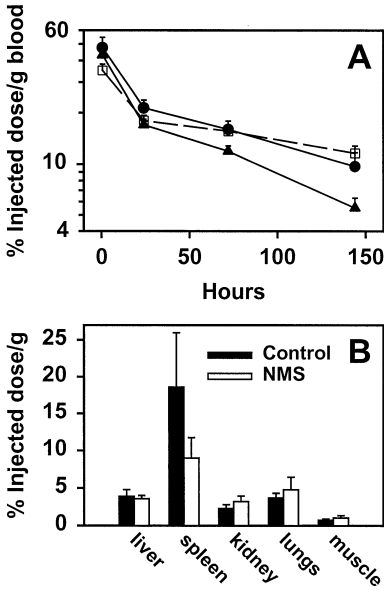

Fig. 7. A, B Effect of co-injected CF-1 mouse serum on biodistribution of ^{125}I-labeled IgG2a in NCI SCID mice. **A** Blood clearance in groups of 5 animals injected i.v. with ^{125}I-IgG2a (1F5), with (*filled circles*) or without (*filled triangles*) 0.1 ml normal CF-1 serum. The IgG2a concentration in this batch of CF-1 serum was 845 μg/ml. Means and standard deviations are shown. For comparison, the blood clearance rate of an IgG1 in mice of the same strain is also shown (*open squares*). **B** Tissue distribution of the radiolabel in the same mice, dissected at the time of the last bleeding (144 h). The group injected with normal CF-1 serum is designated *NMS* (normal mouse serum). Means and standard deviations are shown. Note that CF-1 serum delays blood clearance and inhibits the high level of IgG2a uptake in the spleen

healthy, while all animals not co-injected died ($P < 0.01$; Fisher's test). Results of treatment with ^{111}In-IgG1 are also shown in Fig. 5, for comparison: the MTD appears to be between 300 and 400 μCi in this figure, but many mice were subsequently injected with 350 μCi without death of the mice. Therefore, the MTD with ^{111}In-IgG1 may be slightly higher than that with ^{111}In-IgG2a plus CF-1 serum.

Levels of endogenous IgG2a in outbred CF-1 mice

Since we have used normal CF-1 sera as a convenient and inexpensive source of normal mouse IgG2a, it was important to determine the level of IgG2a in such sera. Individual sera were tested from 10 female retired breeders, 10 male retired breeders, and 11 female non-mated mice 6 months of age. Each group consisted of cage-mates who were received at the same time. Four pools of sera from groups of 13–50 female retired breeders or non-mated mice were also tested. The data showed striking heterogeneity between individual mice. More specifically, for 10 female retired breeders the IgG2a concentrations ranged from 11 to 1,921 μg/ml, a 175-fold range, with a mean of 360 μg/ml. Except for the one mouse having the very high concentration, the second highest concentration was only 362 μg/ml. Three of

these mice had concentrations < 30 μg/ml. For 10 male retired breeders, the range was 155–1,024 μg/ml, with a mean of 457 μg/ml. For 11 female non-mated mice 6 months of age, the range was 55–505 μg/ml, with a mean of 312 μg/ml. Pools of sera from 13–50 females naturally showed somewhat less variation. Five pools were tested, 4 from retired breeders and 1 from non-mated females of approximately the same age. The IgG2a concentrations were 396 μg/ml, 807 μg/ml, 845 μg/ml, 895 μg/ml and 1,860 μg/ml. For the pool with a concentration of 396 μg/ml (obtained from approximately 40 mice), an injection of 0.1 ml per mouse would contain only 40 μg, which, as shown, is not sufficient to adequately block rapid clearance of injected IgG2a. Therefore, even a large pool may contain levels of IgG2a that are too low for the purpose intended. Such variation is not surprising, considering the variation between individual mice, and the fact that 3 of 10 individual CF-1 female retired breeders had IgG2a concentrations of < 30 μg/ml. We conclude that normal mouse serum may be a useful source of irrelevant IgG2a, but that it is essential to determine the actual concentration in each batch.

Discussion

The results presented demonstrate, in general, the importance of considering the factor of IgG blood clearance in experiments with immunodeficient mice. While most of the experiments utilized NCI SCID mice, in which IgG2a clearance is only moderately accelerated, the data obtained with Taconic ICR SCID mice should be emphasized. Most of these mice (21 of 25) cleared injected IgG2a very rapidly: more than 50% was cleared within 30 min, and approximately 90% in 24 h. This blood clearance therefore represents a major obstacle to the use of IgG2a Ab for therapy or for other purposes in these animals (at least at low protein doses). The heterogeneity between individual mice is also notable, in that 4 of the 25 ICR mice tested showed normal clearance of injected IgG2a. Similar results were previously described with nude and *nu*/+ mice. It is very likely that uptake is via the CD64 Fcγ receptor, since endogenous levels of IgG2a are extremely low, since it did not occur with IgG1 Ab or with $F(ab')_2$ fragments of IgG2a Ab, and since the organ with the highest uptake per gram was the spleen.

There is, however, a basic difference between nude and SCID mice in that, in those mice that do not display rapid IgG2a clearance, the explanation appears to be different. In most of the nude mice with slow or normal clearance, endogenous IgG2a concentrations were high, and the level of endogenous IgG2a was inversely correlated with the clearance rate [18]. In contrast, SCID mice with slow clearance of IgG2a, as well as mice with rapid clearance, had extremely low levels of IgG2a and IgG2b (with the exception of one mouse, of 30 tested). Very low IgG levels is an expected phenotype of the *scid* mutation [2]. Therefore, the reason for slow clearance in some SCID mice remains to be explained. The most likely possibility is that the presumed mechanism of uptake, the CD64 Fcγ receptor, is deficient in these mice. While CD64 is not expected to be directly affected by the *scid* mutation, the level of CD64 expression on macrophage-lineage cells is dependent in part on production of lymphokines such as interferon and IL-10 [8, 23, 27], which are likely to be present at abnormally low levels in immunodeficient mice. CD64 is also induced on neutrophils by interferon or G-CSF [8]. Therefore, it seems possible that the immunodeficiency of SCID mice may result in low expression and/or function of CD64. In support of this hypothesis, Yoshida et al. [29] showed that the lack of lymphocytes in SCID mice caused deficient activity of splenic dendritic cells in some functional assays, although they did not examine CD64 expression. In addition, it seems possible that the absence of IgG2a in SCID mice may cause the atrophy of the receptor that normally binds this ligand. This question is difficult to address because Ab to mouse CD64 have not been described.

The use of normal IgG2a or CF-1 sera to block IgG2a clearance has been fairly effective, but large amounts of IgG2a are required to totally block this uptake. From Fig. 6B, it appears that 83 μg IgG2a per mouse was sufficient for reasonably effective inhibition over a period of 3 days, in Taconic ICR SCID mice, which were the strain of SCID mice that displayed the fastest blood clearance. A dose of 28 μg was inadequate. Based on previous studies with nude mice [18], it appears that higher IgG2a doses are required in nude mice in order to have the same effect. In nude mice, 100 μg IgG2a had a weak effect, and 350 μg was required to produce strong inhibition. This difference may depend on the level of CD64 Fc receptor expression in the various mouse strains, and is consistent with other evidence, all indirect, that the level of Fc receptor expression may be lower in SCID mice than in nude mice. Use of normal CF-1 mouse serum as the inhibitor is convenient, but the limitations of this approach must be recognized. With the injection of 0.1 ml undiluted serum per recipient mouse, the amount of IgG2a injected is just sufficient for the purpose intended. Of the 5 large pools of CF-1 sera we have evaluated, 3 had IgG2a concentrations of 800–900 μg/ml, so the amount injected would be approximately 85 μg. One of the pools had approximately 1.8 mg/ml, but the other had only 396 μg/ml. Such variation is consistent with the marked heterogeneity between individual mice, as documented above. It was striking that 3 of 10 8-month old retired breeder female mice had IgG2a concentrations < 30 μg/ml, and that the moderately high average IgG2a concentrations, for groups of 10 mice, were largely due to 1 or 2 mice in the group having very high concentrations of 1–2 mg/ml. Therefore, any mouse serum to be used for this purpose must be assayed for its IgG2a content. These IgG2a concentrations are consistent with those reported previously in various mouse strains [5, 10, 15]. Although we previously reported a value of 6.7 mg/ml in

CF-1 mice [18], which was one of the reasons we used CF-1 sera in the present study, that value was determined in a single experiment, using a pool of sera from a few mice, and is either inaccurate or unrepresentative, based on the data described here. IgG2a concentrations might possibly be raised by immunization of the mice, although the immunization method used by Natsuume-Sakai et al. [10] was not consistently effective. Another approach would be to use ascites of an IgG2a-producing hybridoma, which would be a excellent source of normal mouse IgG2a.

The ELISA to determine IgG2a and IgG2b concentrations may be affected by the particular Ig allotype being detected. There are at least 12 alleles of IgG2a (*Igh-1*), with major antigenic differences between some of them [13, 28]. The anti-IgG2a antiserum used, from Nordic Laboratories, is stated by the supplier to react with most allotypes, including types *Igh-1a* and *Igh-1b*, which are 2 of the most different. To investigate this point experimentally, C57BL/6 Ab were tested in the ELISAs: the IgG2a reacted as well as the BALB/c Ab, while the IgG2b reacted approximately 3-fold less strongly than the BALB/c Ab. Although this difference is significant, it does not affect the conclusions of this study. The other strains of interest for this study are CF-1 and ICR. These are outbred, and we do not know the *Igh* allele or alleles present. However, by testing >10 individual mice of these strains, it was demonstrated that they react well in the assays for both IgG2a and IgG2b. Again, while the absolute concentrations calculated could be slightly inaccurate, such variation would not affect the conclusions of this study. Furthermore, the IgG2a ELISA was previously tested with mouse strains having *Igh-1* alleles *a*, *b*, *c*, *e*, *g* and *h*, and reacted well with all of them [18]. Similarly, the IgG2b ELISA reacted well with mouse strains having the *Igh-3* (IgG2b) alleles *a*, *b*, and *e*, which were all that were tested (unpublished data).

Finally, it seems surprising that this effect was not described previously, since experiments in which it should have been manifested have been performed many times. The same was true for nude mice. In the case of nude mice, we previously noted that similar results were independently reported by 2 other laboratories [18]. More recent results with nude mice by van Gog et al. [25] and Press et al. [16] are also consistent. While the results of van Gog et al. [25] with nude mice are generally consistent with our data, one apparent discrepancy should be noted. They found that levels of IgG2b rather than IgG2a were better correlated with rapid blood clearance of injected human IgG1. In all of their mice, IgG2a levels were extremely low. This is a minor inconsistency, considering that: (1) mouse IgG2a and IgG2b are very similar subclasses in all respects, having developed as a recent gene duplication in the *Mus* lineage, and not present in rats [3]. They have similar functions, and similar physiological control. In general, levels of IgG2a and IgG2b are strongly correlated (unpublished data). (2) IgG2b binds to CD64, although less strongly than IgG2a [24, 26]. In conclusion, this effect may not be recognized, especially if Ab blood clearance is not monitored, and it may have contributed to false-negative results in various Ab localization and therapy experiments.

Acknowledgements We are grateful to Philip Andrews and Nicholas Kumburis for assistance with radiolabeling, and to Linda Reed and Adriane Rosario for assistance with the animal experiments. This work was supported in parts by grants from the National Institutes of Health (CA87059) and the Department of Energy (ER63191).

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