Immunology 1995 **84** 159–163

Unaltered immunoglobulin expression in hybridoma cells modified by targeting of the heavy chain locus with an integration vector

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SUMMARY

Chimeric antibodies against the murine T-cell antigen Thy-1.2 were generated in amounts sufficient for *in vivo* studies by substituting the constant gene segments via homologous recombination in the hybridoma cell. We show that an integration vector targets the heavy chain locus at high frequency even in a non-isogenic situation. Using this vector type, for the first time expression rates were obtained that were identical to the parental hybridoma. The use of the *gpt* selection marker seems to be crucial for efficient expression, and may overcome a recently claimed drawback of vector integration. A chimeric antibody produced by gene targeting was characterized *in vitro* and *in vivo*.

INTRODUCTION

To specifically design the effector functions of antibodies (Ab) and to reduce anti-antibody induction in clinical Ab therapy, chimerization and humanization strategies have been developed (review in ref. 1). Recombinant Ab are mostly expressed in non-producing myeloma cell lines transfected with immunoglobulin gene constructs. The production rates of these transfectants, however, being considerably lower than those of hybridoma cells, are a severe limitation for application in human therapy or in preclinical animal studies (ref. 2 and references within). It was assumed that the differential expression of endogenous and transferred immunoglobulin genes in B cells may be due to the lack of some *cis*-activating elements, e.g. the immunoglobulin 3′ enhancers,[3,4] in the commonly used expression vectors. However, the IgH 3′ enhancer had only a moderate effect on the expression level and could not restore the production rate of a hybridoma.[2] In contrast, very efficient expression of chimeric Ab can be obtained by introducing, by homologous recombination, the desired human constant (C) gene segments into the immunoglobulin loci of the hybridoma cell of interest thereby leaving all regulatory elements intact. Furthermore, this system is very time saving, as the variable (V) genes do not have to be isolated from the hybridoma and one recombination vector can be used for all specificities.

There are two types of recombination vectors:[5] replacement vectors, which mostly carry a two-sided homology flank neighbouring the heterologous region, and integration vectors, which usually contain one homology flank within which the construct is linearized, thus giving rise to a duplication of the target sequence. The exchange of C exons in the immunoglobulin loci has been achieved by using integration[6,7] as well as replacement constructs.[8–10] Integration vectors may have the advantages that they do not need a 3′ flank, which has to be matched to the isotype of the hybridoma, and that the requirement of just one cross-over renders them less sensitive to base pair mismatches.[11] Thus, targeting between sequences of different mouse strains should occur with higher fidelity and frequency.[11]

We have previously shown that chimeric Ab with human Fc regions and specificity for the murine pan T-cell antigen Thy-1.2 are capable of efficiently recruiting effector mechanisms in mice.[12] We then set out to study the immunosuppressive efficiency of these Ab with regard to skin graft survival in a preclinical mouse model. This required Ab amounts that could barely be provided in conventional transfection systems. So far, the expression rates of cell lines modified by gene targeting at the immunoglobulin loci are mostly inferior to those of the parental hybridomas, by at least a factor of two.[6,9,10] It was postulated that only replacement recombination provides the possibility to retain the original production rate.[13] Here we report on the generation of partially chimerized anti-Thy-1.2 Ab for *in vivo* studies; we show that an integration vector is actually highly efficient in a non-isogenic situation, and can produce cell lines that equal the expression level of the parental hybridoma.

Received 29 June 1994; revised 31 August 1994; accepted 2 September 1994.

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MATERIALS AND METHODS

Vector construction

The human IgG1 C region was ligated as a 2·9 kb *Eco*RI–*Pvu*II fragment[14] into *Eco*RI–*Bam*HI-digested pSV2gpt.[15] A 2·3 kb

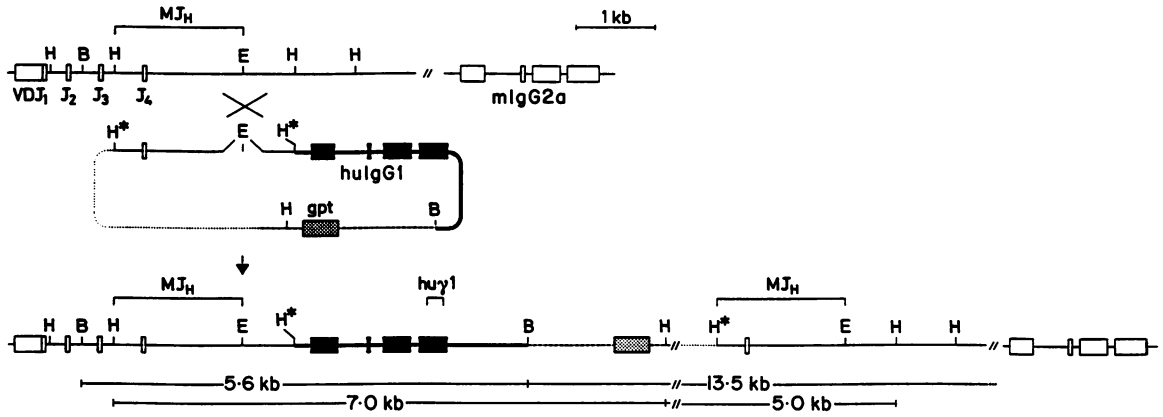

Figure 1. Genomic situation of the functionally rearranged IgH locus in the hybridoma MmT1, and predicted integration pattern induced by the vector pSVgpt–huγ1-A4. The hybridization probes are shown as brackets, the hybridizing fragments as bars. Open and closed boxes indicate mouse and human coding exons, respectively. Broken lines, *gpt* expression unit; stippled lines, pBR sequences. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; the asterisk denotes an inactivated site.

*Hin*dIII fragment from the mouse μ intron was cloned into the *Eco*RI site of this construct, giving rise to pSVgpt–huγ1-A4. The construction of pSVgpt–huγ3-A4 was based on a 2·9 kb *Eco*RI–*Sph*I fragment carrying the human IgG3 C segments.[16] The vector design is shown in Fig. 1.

Cell-culture techniques

MmT1 is an AKR-derived hybridoma (γ2a/κ) with specificity for the murine Thy-1.2 antigen.[17] It was grown in RPMI-1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. For transfection, 2×10^7 cells were suspended in 700 μl RPMI-1640 and mixed with 20 μg of the vector, which had been linearized with *Eco*RI. The cells were pulsed in a BioRad genepulser apparatus (220 V, 500 μF; München, Germany), kept at 0° for 10 min, and plated at a density of 10^4 cells/well in microtitre plates. After 48 hr, selection with 250 μg/ml xanthine, 15 μg/ml hypoxanthine and increasing amounts of mycophenolic acid (2 μg/ml final concentration) was initiated.

Immunological and in vivo *methods*

Supernatants of stably transfected clones were screened by enzyme-linked immunosorbent assay (ELISA) using goat anti-human IgGFc as capture Ab and peroxidase-labelled goat anti-human IgGFc as detection Ab (Dianova, Hamburg, Germany). The colour reaction was developed using o-phenylenediamine and measured in an ELISA reader at 405 nm. For the detection of mouse immunoglobulin, goat anti-mouse IgGFc was used. Quantitative ELISA were performed with purified human or mouse immunoglobulin as calibration proteins.[2] Alternately, Ab concentrations were determined by inhibiting binding to an anti-idiotypic Ab by biotinylated MmT1. This allowed quantification regardless of the isotypes involved. For Western blotting, Ab purified using protein G–Sepharose affinity chromatography was analysed using 8–15% SDS–polyacrylamide gradient gels. After electroblotting the proteins were detected by peroxidase-conjugated goat anti-human IgGFc.

Competitive binding studies were performed using a FACScan (Becton Dickinson, Heidelberg, Germany) calibrated with quantitative fluorescein microbead standards.

T-cell depletion *in vivo* was tested by injection of C57BL/6 mice with 400 μg of Ab intraperitoneally. Tail vein blood was taken on day 3. Blood cells were double-labelled with anti-CD3/anti-CD4, anti-CD3/anti-CD8 or anti-human IgGFc/anti-mouse μ and quantified in the FACS.

Hybridization procedures

Genomic DNA was prepared as described elsewhere.[18] Ten micrograms of DNA digested with various restriction enzymes was run on 0·7% agarose gels and blotted on Genescreen membranes (Du Pont, Boston, MA), which were hybridized with probes ^{32}P-labelled by random priming,[19] and washed under stringent conditions. The probe MJ_H is a 1·6 kb *Hin*dIII–*Eco*RI fragment including the mouse J_H4 segment; huγ1 is a 238 bp polymerase chain reaction (PCR) product derived from the human IgG1 C_H3 exon. (Fig. 1). Fluorescence in *in situ* hybridization was performed essentially as described elsewhere,[20] using a digoxigenin-labelled probe from the μ intron.

RESULTS

High-yield expression of recombinant anti-T-cell Ab following gene targeting with integration vectors

To study the T-cell depleting potential of human isotypes in a mouse model, the anti-Thy-1.2 Ab MmT1 was partially chimerized. The murine IgG2a C exons in the hybridoma MmT1 were exchanged with the human IgG1 or IgG3 region by homologous recombination. The insertion vectors pSVgpt–huγ1-A4 and pSVgpt–huγ3-A4 (Fig. 1) contain the human C exons, a murine 5′ homology flank and the *Ecogpt* marker. They were linearized within the homology region prior to transfection. Targeting events (Fig. 1) were enriched by selection with mycophenolic acid and detected by ELISA. Homologous recombination occurred at a frequency of 0·5%.

About 75% of the targeted clones continued to express the original mouse isotype. As suggested by Southern blot data (see below), this may be explained by the existence of at least two functional IgH copies. As homologous recombination is a rare

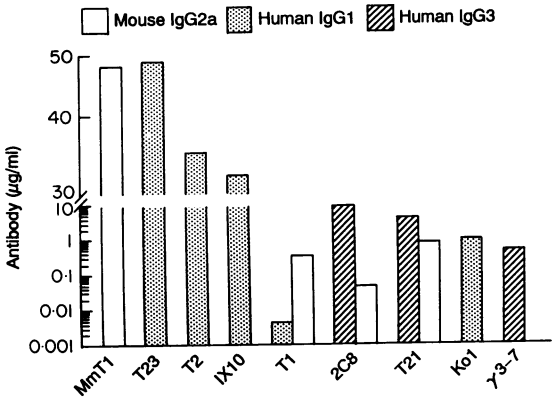

Figure 2. Ab production by selected cell lines generated by homologous recombination. Clones that did and did not coexpress the original mouse isotype are included. For comparison, the production rates of the parental hybridoma MmT1 and of the clones Ko1 and γ3–7 are also shown. The latter two transformants were obtained by conventional cotransfection of an H and L chain construct with MmT1 specificity into the non-producer cell line Sp2/0.[12]

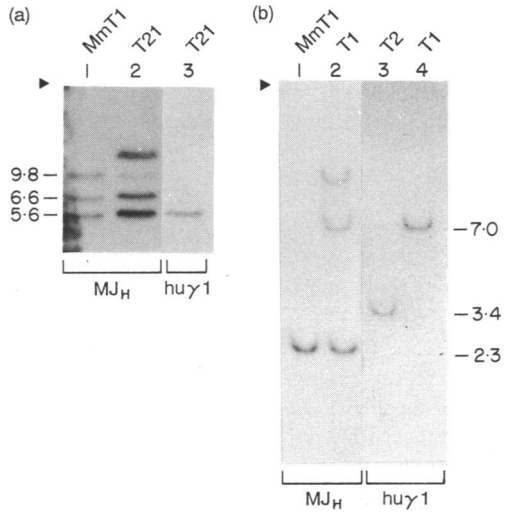

Figure 3. Southern blot analyses of representative clones. DNA from the indicated clones was digested with *Bam*HI (a) or *Hin*dIII (b) and hybridized with the indicated probes. The migration start points are marked by arrow heads. Sizes are given in kb. The 2·3 kb band in lane 1 of (b) represents all three alleles of MmT1 (Fig. 1) and did not disappear upon homologous recombination due to the presence of the aberrantly rearranged alleles, which remained unaltered.

event, clones only secreting the chimeric H chain are probably the result of a loss of the non-targeted copy. We could show, by sandwich ELISA, that the cells expressing two isotypes also secreted hybrid Ab that were composed of the H chains of two different species. Thus, formation of disulphide bridges between mouse IgG2a and human IgG1 and even IgG3 H chains is possible.

The amounts of Ab secreted were measured by quantitative ELISA or by protein determination after Ab purification. Even though there was considerable variation between different clones, in the high-level producers the IgG1 expression was in the range of the parental hybridoma (Fig. 2). Also, the transcript levels were comparable to those of MmT1 (data not shown). In the IgG1 low producers, also the coexpressed murine isotype was produced at low level. This may suggest a limitation of some *trans*-acting factors. Surprisingly, production of human IgG3 was consistently lower. However, this was also observed in conventional transfectants (γ3–7 in Fig. 2) and may be due to a less efficient transport of this isotype from the cell, as much protein could be detected in ELISA using lysates of IgG3-secreting cells.

Genomic situation in the targeted clones

MmT1 contains three H chain alleles (Fig. 3a, lane 1).[21] The 9·8 kb *Bam*HI band represents the functional allele, and the 6·6 kb fragment the aberrantly rearranged allele from the spleen cell; the 5·6 kb band originates from the fusion partner (our unpublished data). T21 was a clone having undergone an expected integration event. There was a novel 5·6 kb fragment that comigrated with that of the fusion partner and which was also detected by the huγ1 probe and a 13·5 kb band resulting from the duplication (Figs 1 and 3a, lanes 2 and 3). However, the functional allele had not disappeared but had just become weaker, which confirmed the existence of multiple gene copies. An *in situ* hybridization was performed to visualize the copies within the cell. We could, however, only detect three spots located on different chromosomes. This suggests that the copies of the functionally rearranged allele are separated by less than 100 kb.

We have demonstrated that integration vectors can initiate replacement-like events involving illegitimate recombinations.[7] We then examined 12 independent clones by Southern blotting and found that six showed this unexpected recombination pattern. For example, the clone T1 showed the predicted 7·0 kb *Hin*dIII fragment hybridizing with MJ_H, as well as with huγ1, but not the 5·0 kb band, which would be indicative for vector integration (Figs 1 and 3b, lanes 2 and 4).

It was postulated that gene replacement results in higher expression rates than vector integration, because it was found that replacement events separated the selection marker from the vector sequence and because a close proximity of the selection marker may exert an adverse effect on immunoglobulin transcription.[13] In some of our clones, for example T1 or T23, the presence of the 7·0 kb *Hin*dIII band indicated that the flank, the human C region and the *gpt* gene had not been separated from one another (Fig. 3b). The linkage was maintained irrespective of having undergone a replacement-like event. In accordance with the above-mentioned study,[13] T1 produced chimeric immunoglobulin in the range of just a few ng/ml, but other transformants such as T23 produced the human isotype at a rate identical to the parental hybridoma (up to 50 μg/ml). On the other hand, there were clones where the *gpt* gene had been integrated into an ectopic position, which was confirmed by various restriction digests and with several hybridization probes. Figure 3b (lane 3) shows the hybridization of *Hin*dIII-digested T2 DNA with huγ1, which detected a fragment of only 3·4 kb, although the corresponding vector sequence encompassing the C region and the *gpt* expression cassette spanned 4·7 kb. Despite the distant location of the selection marker, however, the production rate of T2 was reduced to about 70% compared with that of MmT1. Thus, the

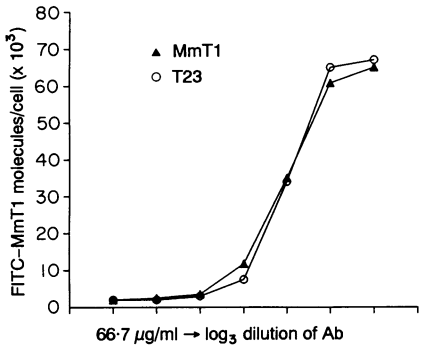

Figure 4. Inhibition of binding of FITC-labelled MmT1 to lymph node cells by serially diluted T23 or MmT1.

expression of chimeric immunoglobulin seems to be independent of the vicinity or absence of the *gpt* gene.

Characterization of the recombinant Ab *in vitro* and *in vivo*

For the *in vivo* studies the human IgG1 chimeric Ab T23 was selected, because IgG1 optimally activates murine effector mechanisms[12] and because this clone is a high-level producer and secretes no mouse Ab. The integrity of the Ab was confirmed by Western blotting (data not shown). The human C_H1 domain was shown to be able to pair with the original mouse κ chain. The antigen-binding avidity was tested in a competition assay. Binding of FITC-labelled MmT1 to Thy-1.2 on lymph node cells was inhibited by serially diluted unlabelled T23 or MmT1. T23 exhibited the same avidity as the original MmT1 (Fig. 4).

T-cell depletion induced by T23 in mice was very efficient. Only 0·9% T cells were found in the peripheral blood after Ab treatment (in comparison to 30% found in normal mice). This finding paves the way to assessing the immunosuppressive potential of T23 in the skin graft model.

DISCUSSION

To generate chimeric Ab for application in a mouse skin graft model, we introduced the desired alteration into the IgH locus of the hybridoma cell line by gene targeting. Our aim was to design a universal recombination system for the chimerization of IgH chains that should be applicable to any hybridoma regardless of the isotype and the mouse strain from which it is derived. Therefore, we used an integration construct, since this vector type needs no 3′ flank and yields higher recombination frequencies and fidelities when sequences non-isogenic to the vector flank are targeted.[11] Here we report for the first time the successful recombination between immunoglobulin sequences of different mouse strains (hybridoma from AKR, vector from BALB/c) and its application for the generation of a partially chimerized Ab for preclinical animal studies. The recombination efficiency (0·5%) was even higher than that reported for another comparable integration vector that was targeted to an isogenic IgH locus.[9] In some cases the integration vector promoted replacement-like events, but these events also mainly gave rise to functional chimeric Ab.

So far, the hybridoma cell lines generated by integration as well as replacement constructs have shown expression levels somewhat lower than those of the parental hybridomas.[6,9,10] Only in the case of gene replacement was the original production rate restored.[8,13] It was claimed that the selection marker which is posed into close proximity to the immunoglobulin expression unit upon vector integration, and which is transcribed from a promoter of its own, might interfere with immunoglobulin transcription.[13] This assumption does not hold true for our system, as in some clones (such as T23) the selection marker had been integrated into the IgH locus, but the expression rate of the parental hybridoma had remained unaltered. Conversely, other clones displayed a reduced production rate, although the selection marker had been separated from the immunoglobulin region. This discrepancy with the previous report[13] might be explained by the inclusion in our constructs of the *gpt* selection marker, instead of the *neo* gene which has been utilized in all other immunoglobulin recombination vectors described so far. Whether or not the original production rate can be achieved is not dependent on the nature of the recombination event, but rather on the use of the proper selection marker. We argue that the *gpt* gene does not impair the expression of chimeric immunoglobulin in *cis*, such as it was postulated for the *neo* gene.[10,13] Given the other advantages of integration vectors, the inclusion of the *gpt* marker renders this vector type even more suitable for routine application.

A problem generally encountered in the targeting of immunoglobulin loci is the frequent occurrence of clones that coexpress immunoglobulin molecules of two species. This finding can be explained by the existence of at least two copies of the functionally rearranged allele. In contrast to other investigators who claim that this is an exceptional feature,[10] we assume that the existence of multiple copies might be common to high-producing hybridomas, since it was observed in a variety of hybridomas[6,7,10] including a rat hybridoma.[7] Our *in situ* hybridization experiments suggest that the different copies are situated on the same chromosome.

Taken together, homologous recombination provides an efficient and convenient method for Ab chimerization. The expression levels obtained have been exceeded only by transfecting amplifiable vectors into non-producer cell lines.[22,23] However, gene targeting may be preferable because of the ease and rapidity of manipulation circumventing the need to isolate V genes, to perform selection schemes over periods of months, and to use toxic drugs.

ACKNOWLEDGMENTS

We wish to thank Dr L. Hood for providing the human C gene segments, Dr E. Kremmer for the cell line MmT1 and Dr M. Reth for the gift of the murine μ intron fragment. Expert technical assistance by A. Kardinal is gratefully acknowledged.

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