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HEAVY CHAIN VARIABLE AND CONSTANT REGION ALLOTYPES IN SINGLE RABBIT PLASMA CELLS*

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Abstract—Spleen cells from rabbits heterozygous for both a variable region (group *a*) allotype and an allotype characteristic of the constant region of IgG (group *d* and group *e*) or the constant region of IgM (*Ms*4 or *Ms*6) were examined by double staining with fluroescent antiallotype sera. The results indicate that one chromosome may contribute a variable region gene while its homologous chromosome may contribute a constant region gene to the synthesis of a single H chain. Such an interaction appears to be responsible for the synthesis of about 1 per cent of the γ and μ chains.

INTRODUCTION

Allotypic markers have been shown to exist in the constant region of the γ-chain of rabbit immunoglobulins (Dubiski, 1969; Mandy and Todd, 1968, 1970). Two groups of specificities have so far been detected. The allotypes *d*11 and *d*12 are correlated with an interchange of methionine for threonine at the position amino terminal and adjacent to the inter-H chain bond in the hinge region (Bahl *et al.*, 1969). The allotypes *e*14 and *e*15 are correlated with an interchange of threonine for alanine closer to the carboxyl terminus at position 309 (Appella *et al.*, 1971). The genes controlling these allotypes are linked to the genes controlling the group *a* allotypes (Dubiski, 1969; Mandy and Todd, 1968, 1970). The group *a* allotypes are shared by the H chains of IgG (Stemke, 1964), IgM (Todd, 1963), IgA (Feinstein, 1963) and IgE (Kindt and Todd, 1969) and correlate with characteristic sequences in the variable part of the heavy chains (Fleischman, 1971; Koshland *et al.*, 1969; Mole *et al.*, 1971; Prahl and Porter, 1968; Wilkinson, 1969a).

Studies have been performed to establish whether individual molecules synthesized by rabbits heterozygous for the group *a* allotypes (variable region) and the group *d* or *e* allotypes (constant region) of the heavy chain always express the allotypic markers determined by the same (paternal or maternal) chromosome. The results indicate that this is generally the case, but the existence of a small proportion (less than 5 per cent) of recombinant heavy chains could not be excluded (Dubiski, 1969; Kindt *et al.*, 1970; Landucci-Tosi *et al.*, 1970).

The problem of detecting small percentages of heavy chains that carry an unusual combination of allotypes has been recently attacked by Landucci-Tosi and Tosi (1973). In the present work, recently developed immunofluorescence techniques (Pernis *et al.*, 1970; Ploem, 1969), which allow an unequivocal detection of two antigenic specificites in single cells, have been applied to this problem. The advantages of this approach derive from the fact that the immunoglobulins contained in the cytoplasm of a single plasma cell constitute a homogeneous sample. By examining a sufficiently large number of cells, the presence of infrequent allotypic combinations can accordingly be detected, and their frequency quantitated with considerable precision. Furthermore, this technique has made possible the study of the association between the group *a* allotypes and some recently discovered allotypic markers that appear to be carried by the constant portion of the rabbit μ chain (Kelus and Pernis, 1971).

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MATERIALS AND METHODS

Rabbits

Rabbits were bred especially for this purpose or chosen by examining their spleen plasma cells by immunofluorescence. For the study of the allotypes specific for IgM, the rabbits were injected once intravenously with sheep erythrocytes or *Proteus vulgaris* X19 cells and sacrificed on the 5th day after immunization. For the study of allotypes specific for IgG, the rabbits received three injections of sheep erythrocytes in a week and were boosted 2 weeks afterwards. The spleens were taken on the 4th day after the booster injection.

Antisera

Antisera specific for rabbit IgG and for rabbit IgM were raised in sheep and made specific as described by Pernis *et al.* (1970).

Antisera specific for the group *a* allotypic markers *a*1, *a*2 and *a*3, were prepared as indicated by Kelus and Gell (1967). Some antiallotype sera, as expected on the basis of the immunization procedure, contain antibodies directed against other immunoglobulin allotypes. These allotypes may not be detectable by immunodiffusion in agar gel, but might contribute to immunofluorescence. Therefore all the antisera against the group *a* allotypes used in this study were absorbed with pools of at least 10 sera from rabbits of other group *a* allotypes made insoluble by cross-linking with ethyl chloroformate. After absorption, all these antisera were tested for specificity in the immunofluorescence system by using them in different pairs to stain the plasma cells of rabbits heterozygous for the group *a* allotypes. These heterozygous rabbits are listed in Table 1. No doubly stained cells were found in any instance. The same antisera were also tested on the cells of rabbits lacking the group *a* allotypes in question, and no positive cells were found.

Antisera to the IgG specific allotypes *d*11 and *d*12 were prepared as described by Mandy and Todd (1968, 1970). Antiserum to the IgG specific allotype *e* 14 was prepared as described by Dubiski (1969). This antiserum was found to contain antibodies to an IgM-specific allotype and was therefore absorbed with an insolubilized serum pool from twelve rabbits lacking the *e*14 allotype. The *d*11, *d*12, and *e*14 antisera (the last after the absorption) were tested on plasma cells of a large number of rabbits. For each antiserum rabbits were found completely lacking the allotypic specificity under study at the plasma cell level.

Antisera to the IgM specific allotypes, *Ms*4 and *Ms*6, were prepared and tested as indicated by Kelus and Pernis (Kelus and Pernis, 1971). These antisera were proved in immunofluorescence to be monospecific and were used without absorption.

Selection of antisera to be used for double staining experiments

In every experiment pairs of antisera possessing the same group *a* and class-specific allotypic determinants were used, so that they could not react one with the other. The combinations of antisera used for double stainings are listed in Table 1.

Conjugation of antisera

The antisera were conjugated with fluorescein or rhodamine isothiocyanate (Baltimore Biological Labs., Baltimore, Md.) by the method of Cebra and Goldstein (1965) as modified by Amante and Giuriani (1969).

Preparation of cell smears

The spleen was cut into small pieces, teased apart, suspended in phosphate buffered saline, ph 7·2, (PBS) containing 5% bovine serum albumin (BSA) and 0·5% ethylenediaminetetracetic acid (EDTA) (final pH adjusted to 6·8–6·9 with solid Na_2CO_3) and ground with a loose-fitting glass homogenizer. The cells were filtered through a 400 mesh stainless steel gauze and washed twice with the above buffered BSA. The entire procedure was performed in the cold. The cells were counted, resuspended at a concentration of 4–5 million cells per ml, and flattened on slides by means of a Shandon–Elliot cytocentrifuge. The slides were either processed immediately or wrapped in aluminum foil, placed in a plastic envelope, and stored at −70°C for later use.

Staining procedure

The cell smears were fixed for 10–15 min at −20°C in a precooled mixture of 96% ethanol (100 vol) and acetic acid (4 vol) and rinsed three times for 15 min with cold PBS. The first antiserum was then placed onto the cells. After 45 min in a moist chamber at room temperature, the slides were thoroughly washed with PBS. The second antiserum was applied as above. Finally the slides were rinsed again with PBS and mounted in phosphate-buffered glycerol (pH 7·0–7·2). The coverslip was sealed with nail polish.

Microscopy

The preparations were viewed under a Leitz Ortholux microscope equipped with the Opak-Fluor vertical illuminator (E. Letiz GmbH, Wetzlar, Germany). This illuminator allows the selective visualization of fluorescein or of rhodamine, through use of different combinations of filters and dichroic mirrors (Ploem, 1969). For selective visualization of fluorescein, the 4 mm BG 12 exciting filter was used with the 495 nm dichroic mirror and barrier filters K495, plus AL525. For selective visualization of rhodamine, the 4 mm BG38, plus 2 mm BG36, plus AL546 exiciting filters were used with the 580 nm dichroic mirror and barrier filters K580 plus K590. Photographs were taken on Ektachrome high speed daylight 23 DIN film or on Kodak TRI-X Pan 27 DIN film.

RESULTS

Screening of spleen cell preparations from randomly bred rabbits

Spleen cell smears from several randomly bred rabbits heterozygous for group *a* allotypes, as tested by double diffusion in agar gel, were stained with antisera to class-specific allotypes and counterstained with heterologous specific anti-IgG or anti-IgM sera. Animals in which only a proportion of IgG or IgM stained plasma cells contained immunoglobulins of the class-specific allotype were presumed to be heterozygous for that marker.

On the basis of the results of the above screening, and because of the limitations in the available antisera used for double staining (see Materials and Methods), only four out of 30 rabbits gave cells suitable for our study.

Table 1. Allotypic specificities of antiallotype sera used in double staining experiments

Antisera against class-specific allotypes	Antisera against group *a* allotypes
Anti-*d*11: *a*1/*d*12/*b*4	Anti-*a*2: *a*1/*d*12/*b*4
Anti-*e*14: *a*2, *a*3/*e*15/*b*6	Anti-*a*1: *a*3/*e*15/*b*4
Anti-*Ms*4: *a*1, *a*3/*b*4	Anti-*a*2: *a*1/*b*4[a]
Anti-*Ms*6: *a*1/*b*5	Anti-*a*2: *a*1/*b*4[a]

[a]The *Ms* specificities of antisera against group *a* allotypes were disregarded since the purified IgG fractions of antisera were used for immunofluorescent staining.

Presence of unusual allotypic associations in plasma cells from rabbits heterozygous for more than one allotype group

Four different combinations were tested, two for the study of association of IgG-specific allotypes with the group *a* allotypes and two for the study of the same association with regard to IgM specific allotypes.

The cells were stained first with the class-specific antiserum conjugated with rhodamine, and then with the antiserum directed to the allotype controlled by the group *a* allele in repulsion, conjugated with fluorescein. When possible, more than one group *a* antiallotype serum was used. Comparable results were always obtained.

In each case, the vast majority of the cells were demonstrated to contain either the class-specific marker or the group *a* marker in repulsion, but a small number of cells contained immunoglobulins possessing both markers. When possible, double staining for the class specific marker and the group group *a* marker in coupling was also performed. In this case nearly but not all of the cells possessing the class-specific markers also possessed the corresponding group *a* marker. Plasma cells that showed only the class-specific marker could have been either recombinants or cells synthesizing immunoglobulins negative for group *a* allotypes. Therefore the frequencies of cells containing possible recombinant molecules were obtained only from the experiments in which the double staining was done for markers controlled by genes located in repulsion. The cells scored as recombinants were all typical plasma cells and the staining with each of the two antisera was quite strong. The results show that recombinant cells exist in a frequency which may differ in different rabbits. These results are reported in Table 2.

The difference of values in cells from rabbit CAN studied with the unabsorbed and absorbed antisera could be accounted for by the presence in the *e*14 antiserum of antibodies directed against an IgM allotype that was linked in this rabbit to the *a*2 allotype (subsequently observed directly and identified as *Ms*4) and therefore the frequency of recombinants observed with the unabsorbed antiserum represented the sum of the IgG and of the IgM recombinants.

Table 2. Double staining for group *a* and class-specific allotypes controlled by genes in repulsion

Rabbit	Genotype	Stain	Cells counted			IgM/IgG	Allelic ratio		Cells Calculated (a)	Cells Calculated (b)	Percent Recombinants[c] (a)	Percent Recombinants[c] (b)
CAN	$\frac{a1/e15}{a2/e14}$	Fluor. anti-*a*1 Rhod. anti-*e*14	$a1^+e14^-$ 3094	$a1^-e14^+$ 932	$a1^+e14^+$ 28	$\frac{70}{30}$	$\frac{a1}{a2}$	$\frac{70}{30}$	2166	2175	1·3	1·3
		Rhod. anti *a*1 Fluor.anti *e*14	649	283	9				454	660	1·9	1·36
		Fluor. anti *a*1[d] Rhod. anti *e*14[e]	1326	396	9				928	924	1·0	1·0
F	$\frac{a2/d12}{a3/d11}$	Fluor. anti *a*2 Rhod. anti *d*11	$a2^+d11^-$ 1415	$a2^-d11^+$ 1040	$a2^+d11^+$ 25	$\frac{89}{11}$	$\frac{a2}{a3}$	$\frac{70}{40}$	1259	1560	2·0	1·6
GE 102	$\frac{a2/Ms4^-}{a3/Ms4^+}$	Flour anti-*a*2 Rhod. anti-*Ms*4	$a2^+Ms4^-$ 1200	$a2^-Ms4^+$ 298	$a2^+Ms4^+$ 2	$\frac{51}{49}$	$\frac{Ms4^+}{Ms4^-}$	$\frac{37}{63}$	588	507	0·3	0·4
TO 5	$\frac{a1/Ms6^+}{a2/Ms6^+}$	Rhod. anti-*Ms*6 Fluor. anti-*a*2	$a2^+Ms6^-$ 316	$a2^-Ms6^+$ 672	$a2^+Ms6^+$ 2	$\frac{54}{46}$	$\frac{Ms6^+}{Ms6^-}$	$\frac{83}{17}$	145	138	1·4	1·4

[a]Calculated on the basis of the IgM/IgG ratio. These ratios were obtained by double staining with anti-IgG and anti-IgM.

[b]Calculated on the basis of the allelic ratio. These ratios were obtained by double staining with antisera directed against group *a* allotypes in rabbit CAN and F, and by double staining with antisera to the *Ms* allotype and to IgM in rabbits GE 102 and TO5.

[c]The percentage of recombinant cells are based on the cells expressing the group *a* allotype and the immunoglobulin class under study. The IgG or IgM cells expressing a given group *a* allotype have been calculated on the basis of the ratio of allelic allotypes and on the basis of the IgG/IgM ratio.

[d]The anti-*a*1 antiserum was absorbed with a pool of insolubilized antisera from rabbits lacking the *a*1 specificity.

[e]The anti-*e*14 serum was absorbed with a pool of insolubilized sera from rabbits lacking the *e*14 specificity.

DISCUSSION

Our basic finding is that individual plasma cells in rabbits heterozygous for the group *a* allotypes and for an allotype in the constant region of the heavy chains contain immunoglobulins that are specified by genes in *cis* position. This was expected on the basis of previous studies on the composition of serum immunoglobulin molecules (Dubiski, 1969; Kindt *et al.*, 1970; Landucci-Tosi, *et al.*, 1970). However, a small percentage of rabbit plasma cells were seen which contained immunoglobulins carrying a group *a* determinant controlled by a chromosome from one parent and a class-specific allotypic determinant controlled by the homologous chromosome from the other parent. This condition might be the consequence of different causes:

(1) Technical artifacts.

(2) Plasma cells in which both homologous chromosomes are independently active for total immunoglobulin systhesis.

(3) Plasma cells synthesizing immunoglobulins the heavy chain of which is controlled by variable region and constant region genes located in repulsion.

Each of these possibilities is discussed below.

Technical artifacts

Nonspecific staining might occur in occasional cells by one or by both antisera. This possibility is unlikely because all the cells scored were strongly stained and typical of the plasma cell series, in which nonspecific staining under our technical conditions practically does not exist. In fact, double staining for two group *a* allotypes with the conjugated antisera employed in the present study did not show, either in the rabbits of Table 2 or in other animals, a single doubly-stained cell.

The presence in the antisera of antibodies (possibly antiallotype) of unexpected specificity is extremely unlikely for the group *a* antisera, because they did not show double staining in rabbits heterozygous for the group *a* allotypes nor did they stain any cell from animals not carrying the expected allotypes. The presence of unexpected antibodies in the class-specific allotype antisera could not be ruled out in the same way. The absorption with a mixture of different sera should have eliminated these antibodies, and in fact it did eliminate the anti-*Ms*4 antibodies present in the *e*14 antiserum. It is striking that the percentage of recombinant cells found in our rabbits are similar to those found by Landucci-Tosi and Tosi (1973) among rabbit IgG molecules reacting with insolubilized antiallotype sera. The presence of unexpected antibodies would have given a percentage of doubly stained cells unrealistically high, whereas such antibodies should have modified the results in the opposite direction in the detection system used by Landucci-Tosi and Tosi.

Finally, the general concordance of the results obtained with different group *a* allotype antisera and also with different antisera against allotypic specificities of the constant region gives confidence to the interpretation that the observed doubly staining cells really contained the allotypic determinants that were being investigated.

Two chromosomes simultaneously active

Cells in which both homologous chromosomes are independently active for immunoglobulin synthesis, if they indeed existed, would have been doubly stained in the conditions employed in our experiments. They should also have appeared as double producers in double staining for allelic group *a* allotypes. This double staining was done on the cells of all the rabbits listed in Table 2. Yet, as indicated above, not a single double stained cell appeared. In contrast, a total of 75 doubly stained cells were found when the double staining was done with antisera against variable region and constant region allotypes in repulsion. This observation rules out the possibility that the latter were cells in which the phenomenon of allelic exclusion had not occurred. Furthermore, the concordance of our cellular percentages with the molecular percentages of Landucci-Tosi and Tosi would not have been found if our double cells had been cells lacking exclusion, since they would have been synthesizing double molecules not detectable as recombinant molecules in the system used by Landucci-Tosi and Tosi

Cells in which variable region and constant region genes are in repulsion

We consider this to be the most likely explanation of our findings. If the messenger RNAs for the variable region and for the constant region of the heavy chain are synthesized separately, and then joined in the cytoplasm, this event is not unexpected. In fact, it should be much more frequent. If however the heavy chain messenger is synthesized as one unit, as the consequence of the translocation of the V region gene to a position in the chromosome adjacent to a given C region gene (Edelman and Gally, 1967), the occurrence of rare cells which contain heavy chains specified by V region and C region genes located in repulsion, would be the consequence of some kind of recombinational event. This recombination could occur during the emergence of the cell line from the zygote to the observed plasma cell or in a step in the selection mechanism for gene activation. The frequency of these recombinational events with respect to cell division is difficult to establish, since we do not know the number of cell divisions from the zygote to the observed plasma cell. Moreover we do not know if any selective forces operated in the individual rabbits either in favor of or against the recombinant cells.

Another possible mechanism would be a muta-

tion at the somatic cell level from $d12$ to $d11$ or the other way around, or from $e14$ to $e15$ and *vice versa*. This possibility is ruled out by the fact that we did not observe $d11$, $d12$ or $e14$ plasma cells in rabbits not carrying the corresponding gene.

Whatever may be the precise mechanism, the frequencies of plasma cells that appear to be synthesizing recombinant heavy chains in individual rabbits average a little over 1%. It is reasonable to assume that the percentage of recombinant molecules in the serum would reflect the percentage of cells in the lymphoid tissue which synthesize them. Accordingly there should be about 1 per cent recombinant molecules in the serum of a rabbit. This figure is too low to be detected in the immunochemical studies previously performed with the aim of detecting recombinant molecules, but matches fairly well with the results recently obtained by Landucci-Tosi and Tosi with an improved immunochemical procedure (1973).

Some cytological aspects of lymphocytes in culture support the possibility that somatic recombination can occur in these cells (German, 1964). Somatic recombination of immunoglobulin genes has been postulated as a major mechanism for somatic generation of diversity among immunoglobulin chains (Edelman and Gally, 1967). While the results reported here and by Landucci-Tosi and Tosi indicate that recombination may not be a major mechanism, it is interesting nonetheless to find some experimental support for its existence.

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