ISOLATED HAPTEN-BINDING RECEP TORS
OF SENSITIZED LYMPHOCYTES
II. Receptors from Nylon Wool-Enriched
Rabbit T Lymphocytes Lack
Serological Determinants of Immunoglobulin
Constant Domains but Carry the
A Locus Allotypic Markers*

BY ULRICH KRAWINKEL,‡ MATTHIAS CRAMER, ROSE G. MAGE,
ANDREW S. KELUS, AND KLAUS RAJEWSKY

(From the Institute for Genetics, University of Cologne, Cologne, Federal Republic of Germany, the
National Institutes of Health, Bethesda, Maryland 20014, and the Basel Institute for Immunology,
Basel, Switzerland)

In the preceding paper† a class of hapten-binding lymphocyte receptor molecules was
described which lack detectable serological determinants of conventional immunoglobu-
lin constant domains but appear to carry heavy chain variable portions. These receptor
molecules which we call the anti-lg− fraction as well as receptor molecules expressing
class-specific immunoglobulin determinants—the so-called anti-lg+ fraction—are isolated
from hapten-sensitized mouse lymphocytes by means of an immunosorbent. The cells are
specifically absorbed to haptenated nylon mesh at 4°C (1, 2) and released by temperature
shift (2). Subsequently hapten-binding material is eluted from the nylon mesh (3-5). The
material can be titrated in the haptenated phage inactivation (HPI) assay (3-5).† The
anti-lg+ fraction was shown to correlate with the B-cell fraction in the cell input whereas
the anti-lg− fraction correlated with the T-cell fraction. It is therefore attractive to think
that the anti-lg+ fraction represents B-cell receptors for antigen whereas the anti-lg−
fraction consists of antigen receptors of T lymphocytes. This interpretation is in accord
with a large body of experimental work (3-6).†

In the present paper we present a similar analysis of lymphocyte receptors for
antigen in the rabbit. The rabbit is particularly suitable for such a study

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riched mouse T lymphocytes lack serological markers of immunoglobulin constant domains but
1 Abbreviations used in this paper: cap, 6-amino-caproic acid; CFA, complete Freund's adjuvant;
CG, chicken Ig; DNP, 2,4-dinitro-phenyl; DNP-cap-T4, DNP-cap-coupled bacteriophage T4;
GaRig, polyspecific mixture of immunosorbents from goat antisera against rabbit Ig as described
in Materials and Methods; HPI, haptenated phage inactivation; IFA, incomplete Freund's adju-
vant; KLH, keyhole limpet hemocyanin; NIP, 4-hydroxy-5-iodo-3-nitro-phenacetyl; NIP-cap-T4,
NIP-cap-coupled bacteriophage T4; NP, 4-hydroxy-3-nitro-phenacetyl; RTLA, rabbit thymus-de-
rived lymphocyte antigen.
because of the large variety of allotypic markers found on rabbit immunoglobulin (7-9). Most relevant for our investigation is the availability of the α locus allotypic markers which are localized on the variable portion of the immunoglobulin heavy chain and are found on the majority of the antibody molecules in the serum (7-9). Since in the mouse system a class of putative T-cell receptor molecules is found which lack class-specific Ig determinants but express idiotype markers of the heavy chain variable portion (5), it is a clear prediction that we should be able to isolate hapten-binding receptor molecules lacking class-specific Ig determinants but carrying α locus allotypic markers from rabbit T lymphocytes. Preliminary evidence had indicated that such molecules can indeed be recovered from rabbit lymphocytes (4). We here confirm this point and demonstrate in addition that in analogy to the situation in the mouse these molecules appear to originate from T cells.

Materials and Methods

Animals. Randomly bred male and female rabbits, aged 4-10 mo, were obtained from a local breeder.

Xenoantisera against Rabbit Immunoglobulins. The following goat antisera (nonabsorbed) were used in this study; goat 12, anti-Fc of rabbit IgG; goat 148, anti-rabbit IgM; goat 150, anti-rabbit colostral IgA; goats 299, 301, and 304, anti-rabbit kappa-type (Lκ) light chains from normal IgG of b4, b5, and b9 allotype, respectively; and goat 306, anti-rabbit lambda-type light chains (from a c7+/c21+, b5 suppressed homozygous donor). Each of these sera was the final bleeding from a hyperimmunized goat which had received an initial immunization with purified antigen in complete Freund’s adjuvant (CFA) and repeated boosting injections of the same antigen in incomplete Freund’s adjuvant (IFA) over periods ranging from 2.5 (goat 301) to 8 yr (goat 12).

Anti-Allotypic Antisera. Partially inbred rabbits from the colony maintained in Basel were immunized with Proteus vulgaris X 19 and subsequently used for the production of anti-allotypic antisera employing Proteus coated with rabbit antibodies of a defined genetic make-up (7). Animals were selected in a way that the donor and the recipient differed by one allotypic specificity only. Antisera against the allotypic specificities a1 and a2 were raised in a3/a3 homozygous rabbits whereas the anti-a3 sera were raised in a2/a2 homozygous animals. The antisera did not show any cross-reactivity as judged by a sensitive double diffusion test in polyethylene glycol gels. This test can detect IgG in a concentration of as little as 1 μg/ml (10). The anti-Proteus activity in the anti-allotypic antisera did not interfere with the allotypic reaction. An independent set of antisera against allotypes a1, a2, and a3 was kindly donated by Dr. S. Dubiski.

Determination of Allotypes. Preimmune and immune sera (1:3 dilutions) of all rabbits were typed in double diffusion assays in 0.8% agarose containing 3% polyethylene glycol (Merck AG, Darmstadt, W. Germany) for α locus allotypes with several anti-allotypic sera in each case.

Haptens and Antigens. The 6-amino-caproic acid (cap) derivative of 4-hydroxy-3-nitro-phenacyl (NP) was synthesized according to the method of Brownstone et al. (11). 2,4-Dinitrophenyl (DNP)-cap was obtained from Sigma, München, W. Germany. The hapten-protein conjugates 4-hydroxy-5-iodo-3-nitro-phenacyl(NIP)19-chicken gammaglobulin (CG) and DNP2-keyhole limpet hemocyanin (KLH) are described in the previous publication.1

Immunizations. Each rabbit received 1 mg DNP2-KLH or NIP19-CG in 4 ml CFA injected into the footpads, subcutaneously at various sites, and intraperitoneally. In the case of NIP19-CG the animals were boosted 4 wk later with 0.5 mg of hapten-protein conjugate in 4 ml IFA subcutaneously and intraperitoneally. 4 wk after the last injection lymph nodes and spleens were harvested. Single cell suspensions in minimal essential medium (Flow Laboratories, Inglewood, Calif.) buffered with 0.01 M HEPES (Flow Laboratories) and containing 15-20 μg/ml calf thymus deoxyribonuclease II (Roth, Karlsruhe, W. Germany) were prepared as described previously for splenic mouse lymphocytes (3).1

Enrichment of T Lymphocytes. Rabbit lymph node and spleen cells were fractionated according to the procedure of Julius et al. (12) with the slight modifications described previously, i.e., 10 mg/ml bovine serum albumin was used instead of 5% fetal calf serum for washing the nylon wool columns.1 Enrichment of T lymphocytes was verified in two ways: (a) cells were stained with
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fluorescein-isothiocyanate-coupled goat anti-rabbit Ig antibodies (Behring-Werke AG, Marburg/Lahn, W. Germany) as previously described for mouse lymphocytes,1 and (b) cytotoxic assays were performed with a goat antiserum to the thymus-derived lymphocyte antigen (RTLA). This antiserum as well as the appropriate rabbit complement was kindly donated by Dr. B. Cinader. The antiserum and the cytotoxic assays are described in reference 13.

Preparation of Receptor Material on Haptenated Nylon. Spleen and lymph node cells were absorbed to and released from haptenated nylon disks according to the method of Kiefer (3, 14) as described previously for murine lymphocytes (3).1 Receptor material to DNPg-KLH was eluted from DNP-cap-coupled nylon disks with 10^{-3} M DNP-cap. Cells of NIP_{10-CG} sensitized rabbits were adsorbed to nylon disks coupled with the cross-reactive hapten NP-cap and released by temperature shift. Receptor material was eluted with 5 \times 10^{-4} M NP-cap as described in the previous publication.1 Reduction of volume, dialysis, and storage of receptor material were also strictly analogous to the procedures employed to prepare murine receptors (3).

Immunosorbents and Absorptions. The preparation of immunosorbents of xenoantisera to rabbit Ig and of anti-allotypic antisera as well as absorptions of receptor material and serum antibody were carried out as previously described (3). The following immunosorbents were prepared: goat antisera to rabbit IgG (goat 12), IgM (goat 148), and IgA (goat 150) were individually cross-linked with glutardialdehyde (15) and mixed afterwards in equal proportions to yield a polyspecific goat anti-rabbit Ig immunosorbent (GaRIg). Goat antisera 299, 301, 304 (anti-κ), and 306 (anti-λ) were treated in the same way to obtain an immunosorbent against rabbit light chains. Anti-allotypic immunosorbents of three different antisera for each allotypic specificity were individually prepared with Sepharose 4B (16) (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). These immunosorbents were used separately or as mixtures of immunosorbents as stated in Fig. 2.

Haptenated Phage Inactivation (HPI) Assay. NIP-cap- and DNP-cap-T4 bacteriophages were a gift of Doctors M. Becker and O. Mäkelä (17). HPI assays were carried out as previously described (3).1 Receptor activity is expressed in HPI units. Receptor activity (HPI units) = HPI titer of receptor preparation \times volume of receptor preparation (milliliters). The yield of receptor activity is given in HPI units per 10^8 input lymphocytes.1

Results

Isolation of Hapten-Specific Receptor Material from Sensitized Rabbit Lymphocytes. Previous experiments had shown that hapten-binding receptor material can be isolated from sensitized rabbit lymphocytes in the same way as originally described for murine lymphocytes (4). For the present analysis we used four allotypically defined rabbits which were immunized with either DNP_{g-KLH} or with NIP_{10-CG}. The animals were bled, killed, and lymph node and spleen cells were prepared from each individual animal. In three cases the cells were passed over nylon wool columns (12) to enrich for T lymphocytes. For each cell preparation, the fraction of cells sensitive to anti-RTLA antiserum and complement and/or the fraction of cells bearing surface Ig were determined. The data indicated efficient enrichment of T lymphocytes (see Table I). It was found that like in the mouse system (3) 0.5–1.2% of these sensitized lymphocytes bound to and could be released from hapten-coupled nylon disks regardless whether they were enriched for T cells or not. Receptor material was then isolated from each cell batch with the hapten DNP-cap in the case of the DNP_{g-KLH} sensitized rabbits and NP-cap in the case of the NIP_{10-CG} sensitized cell donors. The activity of the various receptor preparations was determined in the HPI assay. The results appear in Table I.

In the case of NIP-specific receptors, the yields were similar to those previously found in the mouse system1 and, most importantly, they were in the same order for unseparated cells and enriched T lymphocytes. In the case of DNP-
specific receptors, the yield from the enriched T-cell population was rather low. In fact, from two other DNP$_s$-KLH sensitized T-cell populations no detectable anti-DNP activity was recovered, whereas from an unseparated cell batch a very active receptor preparation was obtained (data not shown). The reason for this result is unknown. It should be mentioned, however, that the DNP-specific receptor preparation from the unseparated cells could be absorbed to more than 95% on insolubilized class-specific anti-Ig sera. Since this material thus lacked the anti-Ig$^-$ fraction (see below) it was not included in the present analysis.

Two Classes of Receptors from Sensitized Rabbit Lymphocytes: the Anti-Ig$^+$ and the Anti-Ig$^-$ Fraction. The receptor preparations as characterized in Table I were first subjected to a similar analysis as was previously carried out with receptor material isolated from murine lymphocytes (3-5). The material was passed through a variety of anti-immunoglobulin immunosorbents to determine whether the phage-inactivating molecules carry class-specific determinants of immunoglobulin polypeptide chains. The analysis included antisera against the IgG, IgA, and IgM classes of rabbit Ig as well as against k- and $\lambda$-light chains. Humoral antibodies from the lymphocyte donors served as a positive control for the efficiency of absorption. The results appear in Fig. 1. As one would expect, the anti-Ig immunosorbents eliminate virtually all of the activity of humoral antibody. This is true for both the immunosorbents made from antisera raised against whole Ig molecules or Fc fragments of the various heavy-chain classes and for the immunosorbents prepared from antisera specific for isolated k and $\lambda$ chains (Fig. 1 e). However, in the case of lymphocyte-derived receptor material the situation is entirely different. Receptors from the unseparated lymphocyte population can be absorbed from 70 to 80% by the various anti-Ig immunosorbents. The same immunosorbents absorb only a small fraction (10-20%) of receptor activity from material isolated from nylon wool-enriched T lymphocytes in two of the three cases. In the third, the absorbed fraction amounts to approximately 45%. The situation is thus analogous to that encountered in the mouse system: the receptor material obtained from rabbit lymphocytes can be classified into an anti-Ig$^+$ and an anti-Ig$^-$ fraction as our previous data already indicated (4). In addition, T-cell enrichment in the input cell population results in an increased proportion of the anti-Ig$^-$ fraction.
Receptors of the Anti-Ig^- Fraction Carry the a Locus Allotypic Determinants. For allotypic analysis of the anti-Ig^- receptor fraction, the various receptor preparations were first absorbed on insolubilized polyspecific GaRIG sera and subsequently on various immunosorbents prepared from anti-allotypic antisera specific for the a locus allotypic determinants. For each allotypic specificity three antisera were included in the analysis and assayed separately. The results appear in Fig. 2. Inspection of the data reveals a complete consistency between the various experiments. In every single case each of the various batches of anti-allotypic antisera specific for the allotype expressed in the humoral antibody of the cell donor eliminates the bulk of the phage-inactivating activity of the receptor preparation. Immunosorbents prepared from anti-allotypic antisera recognizing specificities of the wrong a locus allotype are inefficient in every single case. The conclusion appears inescapable that the anti-Ig^- receptor fraction, putatively of T-cell origin, carries the a locus allotypic determinants.

Discussion

The present data confirm and considerably extend our previous results on
receptor molecules isolated from rabbit lymphocytes (4). In analogy to the mouse system (3-5)\textsuperscript{3} we find two fractions of receptor molecules, one carrying serological determinants of immunoglobulin constant domains (anti-Ig\textsuperscript{+}) and probably originating from B cells, the other lacking any detectable serological determinants of γ-, α-, μ-, λ-, or k-chain constant parts (anti-Ig\textsuperscript{-}) and probably originating from T cells.

The T-cell origin of the anti-Ig\textsuperscript{-} fraction appears likely on the basis of our cell separation experiments. In receptor material isolated from unseparated rabbit lymphocytes we have in previous (4) and the present experiments always observed predominance of the anti-Ig\textsuperscript{+} fraction ranging from 65 to 75% (and 95%
in an exceptional case, see page 795) of the total activity. When the lymphocyte population was passed through nylon wool columns, a striking enrichment of cells lacking surface Ig but sensitive to an antiserum considered to be specific for T lymphocytes (13) was observed. It appears reasonable, in analogy to the mouse system, to consider these cells belonging to the T compartment. Receptor activity could be recovered from such cell populations in a yield similar to that obtained frequently in the case of unseparated cells, but within this activity the anti-Ig− fraction was now predominantly expressed. In two out of three cases less than 20% of the active molecules were anti-Ig+. In the third case, the anti-Ig+ fraction was slightly below 50% and thus lower than in material isolated from unseparated cells, but higher than in the two other T-cell-derived preparations. Many reasons can be invoked to explain variations of this kind, in particular if one takes into account a case like the one in which the anti-Ig+ fraction in material derived from unseparated cells of a particular rabbit amounted to more than 95% of the total activity (see page 795). If T cells were purified from a cell population of this kind and receptors isolated from those cells, the material would be expected to be heavily contaminated with the anti-Ig+ molecules. Fortunately, situations like this have been exceptional so far and were in particular never encountered in the mouse (3–5). Looking at the overall data which we have obtained in mice and rabbits, it appears therefore very likely that the anti-Ig− fraction is T-cell-derived, although we can not yet prove this point. The overall evidence argues strongly against the possibility that the anti-Ig− fraction is an experimental artifact in the sense of a breakdown product of Ig molecules. This point has been discussed in more detail elsewhere (6). It may suffice to mention in this context the absence of determinants of constant domains of immunoglobulin light chains on the anti-Ig− fraction and the finding of hapten-binding activity in murine receptor preparations being associated with molecules with molecular weights above 10^5 daltons in Sephadex gel filtration (Pharmacia Fine Chemicals, Inc.) and sucrose gradient centrifugation experiments. Undocumented experiments have shown that the latter is also true for the anti-Ig− fraction in the rabbit.

In the mouse, the anti-Ig− fraction was shown to carry idiotypic and fine specificity markers of the heavy chain variable region. As in antibodies these markers were under the control of genes in the heavy chain linkage group (18). The present data seem to establish that in the rabbit the molecules in the anti-Ig− receptor fraction express the a locus allotypic determinants, i.e. again structures encoded by genes in the heavy chain linkage group (7–9). Of course we do not have a formal proof that the determinants which we detect on the anti-Ig− fraction by our immunosorbent analyses are identical to those present on V_h regions of rabbit antibodies. However, the complete consistency of our results which are based on a variety of receptor preparations analyzed with a variety of anti-allotypic antisera strongly suggests that indeed the same genes code for variable portions on humoral antibodies and receptor molecules in the anti-Ig− fraction which we consider T-cell-derived.

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The α locus allotypic determinants as well as the idiotypic determinants detected by our anti-idiotype on C57BL/6 anti-NP antibodies appear to relate to framework residues of the variable region of the Ig heavy chain (9, 19, 20), whereas the fine specificity marker of C57BL/6 anti-NP antibody must be part of its complementarity-determining portion. Since we find all these genetic markers on the anti-Ig- receptor fraction, we conclude that the hapten-binding molecules in this fraction express the entire V region of antibody heavy chains.

The implications of this finding together with that of a complete lack of other determinants of constant parts of known immunoglobulin polypeptide chains in the anti-Ig- receptor fraction for the structure of the T-cell receptor has been discussed in the previous paper and elsewhere (6). In these discussions we have also pointed out the consistency of the present results with those of Binz et al. (21, 22) and with our own idiotypic analysis of T-helper-cell function (6, 23). Also in accord with our results are the recent data of Cazenave et al., who found that activated rabbit T cells express α locus allotypic determinants but lack determinants controlled by the β locus (24). It remains an unresolved puzzle why other workers in careful analyses have failed to detect the α locus allotypes on rabbit T cells (25) and also on antigen-specific helper factors of putative T-cell origin (26), although in the latter case the evidence appeared preliminary. Whereas it is clear that the discrepancy may have trivial technical reasons or may relate to different stages of activation of the cells analyzed, it should be stressed at this point that we have so far no formal proof of the T-cell origin of the anti-Ig- receptor fraction nor of its localization on the cell surface.

The present data support the contention that the immune system generates T-cell receptor molecules which lack known structures of antibody polypeptide chains except one: the variable portion of the Ig heavy chain. It has to be kept in mind, however, that the evidence for and against the presence of light chain variable portions on T-cell receptors are only indirect and/or circumstantial (4). Our own analyses (5) as well as those of Binz et al. (22) have so far failed to detect structures encoded by the major histocompatibility complex on receptor molecules of the anti-Ig- phenotype. It, thus, remains unknown not only whether the VH regions constitute the complete binding site of the anti-Ig- receptor molecules, but also whether these molecules represent the entire receptor structure of the cells from which they originate.

Summary

Hapten-binding receptor material was isolated from sensitized rabbit lymphocytes by a method described previously for murine receptor material (3). The material was separated into a fraction expressing immunoglobulin determinants (anti-Ig+ fraction) and a fraction lacking known class and type-specific determinants of Ig constant domains (anti-Ig- fraction). We present evidence in support of the notion that—in analogy to the mouse system—the anti-Ig+ fraction is B-cell-derived, whereas the anti-Ig- fraction originates from T lymphocytes. Receptors of the anti-Ig- phenotype are found to express α locus allotypic determinants and, thus, appear to carry variable portions of immunoglobulin heavy chains.
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References


