Thymus-Independence of Slowly Metabolized Immunogens
(synthetic antigens/multichain poly(proline)/repeating antigenic determinants/optical configuration)

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Communicated by Herman N. Eisen, July 15, 1972

ABSTRACT  The role of thymus in antibody responses to a series of four synthetic polypeptide immunogens of the general formula: multi-copoly(Tyr,Glu)–poly(Pro)–poly(L-Lys) was investigated as a function of the optical activity of the amino acids composing their structure. Irradiated non-thymectomized and thymectomized SJL mice were injected with thymocytes, marrow cells, or a mixture of both. Each group of recipients was immunized with the following copolymer enantiomers: all L-amino acids; L-amino acids outside and D inside; D-amino acids outside and L inside; or all D-amino acids. The antibody response to the immunogen composed of all L-amino acids was thymus-dependent, whereas the responses to the other three copolymers were all independent of the thymus. Similar cell transfers were performed in DBA/1 mice immunized with multi-copoly(L-Phe,L-Glu)–poly(D-Pro)–poly(D-Lys). This mouse strain produces specific antibodies against the (Phe,Glu) region and against the poly(D-prolyl) region. The immune response to the determinant with only L-amino acids on the outside was thymus-dependent, whereas the response to the inside immunopotent region with only D-amino acids was thymus-independent. Since earlier studies have demonstrated that synthetic polypeptide antigens that contain D-amino acids are poorly metabolized, the thymus-independence of the antibody responses to these multichain synthetic polypeptides that possess repeating antigenic determinants was correlated with the metabolizability of the immunogens or their component determinants.

The phenomenon of cell-to-cell interaction has occupied an important place in studies of the cellular nature of the immune response. Cooperation between thymus-derived and marrow-derived cellular components of the immune system of mice has been demonstrated for humoral antibody responses to several immunogens including heterologous erythrocytes (1–3), serum proteins (4–6), and synthetic polypeptides (7–9). Immunogens have been described recently that elicit humoral antibody responses in heavily irradiated, bone-marrow reconstituted mice without the presence of detectable numbers of thymocytes or thymus-derived cells. These immunogens have been called “thymus-independent antigens,” and include polymerized flagellin (10, 11), pneumococcal polysaccharide (12), Escherichia coli lipopolysaccharide (13), and polyvinylpyrrolidone (13). Since they consist of long chains made up of repeating antigenic determinants (polymerized flagellin contains repeating polypeptide chain subunits), the suggestion has been made that the structural requirement necessary for an immunogen to be independent of the thymus is the presence of a repeating antigenic subunit (14, 15). This may be a necessary but insufficient requirement for thymus-independence, since several multichain synthetic polypeptide immunogens, all possessing repeating antigenic determinants, require both thymus- and marrow-derived cells for eliciting efficient humoral immune responses in mice (7–9).

It has been postulated that thymus-derived cells function to concentrate antigen (16). It, therefore, seemed possible that in addition to repeating antigenic determinants, slow metabolism might also be required for efficient antibody responses in mice to some immunogens in the absence of thymus cells. In order to investigate this hypothesis, use was made of the fact that immunogens composed of D amino acids are slowly and incompletely metabolized (for review see Gill, ref. 17). This report demonstrates that the immune response to a multichain synthetic polypeptide composed exclusively of D amino acids is independent of the thymus.

The multichain synthetic copolypeptides used in this study can be prepared in such a manner that different parts of the molecule may be built from amino acids that differ in their optical configuration. Thus, as seen in Fig. 1, the multichain copolymer of the general formula multi-copoly(Tyr,Glu)–poly(Pro)–poly(L-Lys) is composed of two moieties: outside and inside, and each of these may be prepared from amino acids of the L or of the D configuration. Earlier studies have shown great differences in the immunogenicity and metabolism of such enantiomers (18, 19). The results reported here show that these materials, or their unique determinants, were either dependent on or independent of the thymus as a function of their metabolizability.

MATERIALS AND METHODS

The immunogens used in this study were four multichain synthetic polypeptides of the general formula multi-copoly(Tyr,Glu)–poly(Pro)–poly(L-Lys), namely, poly(L-Tyr,L-Glu)–poly(L-Pro)–poly(L-Lys), 918; poly(L-Tyr,L-Glu)–poly(D-Pro)–poly(L-Lys) 711; poly(L-Tyr,L-Glu)–poly(L-Pro)–poly(L-Lys), 715; and poly(D-Tyr,L-Glu)–poly(D-Pro)–poly(L-Lys), 713. In addition, the multichain copolymer poly(L-Phe, L-Glu)–poly(D-Pro)–poly(L-Lys), 712, was also used. The synthesis, characterization, immunogenicity, and metabolism of these polymers have been reported (18–20, *).

Inbred SJL and DBA/1 female mice, 9–11 weeks of age, obtained from the Experimental Animal Unit, The Weizmann Institute of Science, were used throughout the experiments. Thymus and bone-marrow cell suspensions were prepared as described (7), and injected in the tail vein of syngeneic recipients exposed to 750 R of 51Co gamma-ir-
radiation. In one experiment, the host animals were thy-
metomized three weeks before irradiation. Each recipient
received either $10^8$ thymocytes or $2 \times 10^7$ bone-marrow cells,
or a mixture containing $10^6$ thymocytes and $2 \times 10^7$ marrow
cells. 24 Hr after cell transfer, each recipient was immu-
nized intraperitoneally with 10 µg of one of the above
immunogens in complete Freund’s adjuvant (Difco Labora-
tories). 2 Weeks later (at the time of peak antibody
titers), the mice were bled and their sera were individually
titered for antibodies by passive microhemagglutination,
with tanned, antigen-coated sheep erythrocytes (7). The
homologous antigens were used for assay of sera of mice
immunized with the four enantiomorphs of poly(Tyr,Glu)-
poly(Pro)--poly(Lys). Since poly(L-Phe,L-Glu)--poly(D-Pro)--
poly(D-Lys) initiates a good antibody response both to the
poly(L-Phe,L-Glu) moiety and to the poly(D-proline) side
chains in DBA/1 mice, the sera of DBA/1 mice immunized
with this polypeptide were titered separately with erythro-
cytes coated with poly(L-Phe,L-Glu)--poly(D-Pro)--poly(D-
Lys) for the response to poly(L-Phe,L-Glu), and with erythro-
cytes coated with poly(D-Tyr,D-Glu)--poly(D-Pro)--poly(D-
Lys) for the specific response to poly(D-proline). As the antibody
titers of sera from irradiated recipients, each injected with thymus
and marrow cells were weak (1:8-1:32), the data are expressed
as the fraction or percentage of recipients showing hemaggul-
tination at serum dilutions higher than that observed in ir-
radiated controls (i.e., at dilutions of 1:8 or higher).

**RESULTS**

In order to elucidate whether poorly metabolized synthetic
polypeptides composed exclusively or partially of D-amino
acids are independent of the thymus in a cell-transfer sys-
tem, the antibody responses to synthetic polypeptide im-
munogens of the formula multi-copoly(Tyr,Glu)--poly(Pro)--
poly(Lys) were studied. This polymer is composed of two
moieties: The outside or terminal peptides of tyrosine and
glutamic acid, and the inside area of poly(proline) side-chains

![Fig. 1. Schematic representation of a multichain copolymer
composed of a backbone of poly(lysine) to which are attached side
chains of poly(proline) (the inside area), elongated with peptides
of tyrosine (or phenylalanine) and glutamic acid (the outside area).]

**TABLE 1. Thymus–bone marrow cell cooperation in the
humoral antibody response of SJL mice as a function of the
optical configuration of the amino acids composing the immuno-
gen multi-copoly(Tyr,Glu)--poly(Pro)--poly(Lys)**

<table>
<thead>
<tr>
<th>Immunogen and assaying antigen</th>
<th>Cells injected per recipient*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^6$</td>
</tr>
</tbody>
</table>
| Poly(L-Tyr,L-Glu)--poly(L-
Pro)--poly(L-Lys) | 0/9 | 0 | 2/24 | 16/16 | 100 |
| Poly(L-Tyr,L-Glu)--poly(D-
Pro)--poly(L-Lys) | Not | 11/15 | 73 | 8/15 | 56 |
| Poly(D-Tyr,D-Glu)--poly(L-
Pro)--poly(L-Lys) | 0/12 | 0 | 16/23 | 70 | 18/28 | 64 |
| Poly(D-Tyr,D-Glu)--poly(D-
Pro)--poly(D-Lys) | 0/14 | 0 | 15/24 | 63 | 16/27 | 59 |
| Poly(D-Tyr,D-Glu)--poly(D-
Pro)--poly(D-Lys)† | 0/8 | 0 | 7/11 | 63 | 8/11 | 72 |

* Fraction and percentage of irradiated recipient mice pro-
ducing detectable antibody titers after cell transfer. 10$^6$ thymo-
cytes and/or 2 x $10^7$ marrow cells were injected per recipient.
Host mice were immunized 24 hr later and bled 2 weeks after
cell transfer.
† Experiment in which thymectomized irradiated recipients
were used.

attached to a poly(lysine) backbone. Four polypeptides were
prepared that differed in the optical activity of their com-
ponent amino acids (18). Thus, the following four enanti-
omorphs of poly(Tyr,Glu)--poly(Pro)--poly(Lys) were obtained:
(a) All L-amino acids, (b) all D-amino acids, (c) L-amino acids
outside and D inside, and (d) D-amino acids outside and L
inside. It is noteworthy that the polymer that has D-amino
acids on the outside and L inside (but more than 90% of the
amino acids are of the L-configuration) behaved similarly to
the polymer with only D-amino acids, both in its immuno-
genicity in rabbits being expressed only at very small doses
(18), and in its slow metabolism (19). This is due to the lack of
dendopeptidases capable of splitting peptide bonds between
two L-proline residues. As every poly(L-proline) chain in multi-
copoly(Tyr,D-Glu)--poly(L-Pro)--poly(L-Lys) is linked to an α-
amino group of lysine on one end and to a peptide composed of
D-amino acids on the other end, no significant digestion of
this polymer can occur within the animal body, similarly to
copolymers composed exclusively of D-amino acids.

The four different enantiomorphs of poly(Tyr,Glu)--poly(
Pro)--poly(Lys) were tested for their ability to elicit a humoral
antibody response in irradiated mice injected with $10^6$
thymocytes, 2 x $10^7$ marrow cells, or a mixture of these two
cell populations. The results of the response frequencies ob-
tained are shown in Table 1. The antibody response to the
copolypeptide composed exclusively of L-amino acids was found
to be thymus-dependent, since the injection of thymus or
marrow cells alone did not generate a significant immune
response, whereas injection of the cell mixture generated
detectable responses in the sera of all the recipients tested.
In contrast, when poly(Tyr,Glu)--poly(Pro)--poly(Lys) was
composed of L-amino acids outside and D-amino acids inside, D
Table 2. Thymus-bone marrow cell cooperation in the humoral antibody response of DBA/1 mice as a function of the optical configuration of the antigenic determinants within the immunogen multi-copoly(l-Phe,L-Glu)-poly(l-Pro-poly(l-Lys)).

<table>
<thead>
<tr>
<th>Antigen used for assay</th>
<th>Cells injected per recipient*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10³ Thymocytes and Marrow cells</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁷</td>
</tr>
<tr>
<td>Poly(l-Phe, L-Glu)-poly-(L-Ala)-poly(l-Lys) [for poly(l-Phe, L-Glu) specificity]</td>
<td>2/15 13 3/19 16 17/23 74</td>
</tr>
<tr>
<td>Poly(l-Tyr, D-Glu)-poly(l-Pro)-poly(l-Lys) [for poly(l-Pro) specificity]</td>
<td>1/15 6 12/19 63 16/23 69</td>
</tr>
</tbody>
</table>

*See the same footnote to Table 1.

In order to test whether an immunogen composed of L-amino acids outside and D-amino acids inside contains a thymus-dependent as well as a thymus-independent immunogenic region, use was made of the fact that DBA/1 mice immunized with multi-copoly(l-Phe, L-Glu)-poly(l-Pro-poly(l-Lys)) generate antibodies specific both for the poly-(l-Phe, L-Glu) and for the poly(l-Pro) portions of this immunogen*. Irradiated DBA/1 mice were injected with 10³ thymocytes, 2 x 10⁷ marrow cells, or a mixture containing 10³ thymus and 2 x 10⁷ marrow cells. The recipients were immunized with poly(l-Phe, L-Glu)-poly(l-Pro-poly(l-Lys)), and their sera were assayed for specific antiopoly(l-Phe, L-Glu) and antiopoly(l-Pro) antibodies. The results summarized in Table 2 indicate that the immune response to the moiety with the L-amino acids outside is thymus-dependent, since only 16% of the recipients injected with marrow cells alone responded, whereas 74% of the mice injected with marrow and thymus cells responded. In contrast, the antibody response to the moiety with D-amino acids on the inside of the immunogen was independent of the thymus, since from 63 to 69% of the recipients injected with bone marrow cells responded irrespective of the addition of thymocytes. These latter observations indicate that synthetic immunogens can be prepared that are composed of thymus-dependent and -independent regions, and support the hypothesis that the immune response to poorly metabolized immunogens, as well as to poorly metabolized antigenic regions of immunogenic macromolecules, is independent of the thymus.

**DISCUSSION**

The humoral immune responses to several immunogens have been shown to require the cooperation of at least two distinct populations of immunocompetent cells (1–9): thymus-derived, antigen-reactive cells and bone marrow-derived precursors of antibody-forming cells. However, exceptions to this experimental observation have recently been described, since antibody responses were obtained to certain immunogens that appear not to involve the thymus-derived component of the immune system (10–13). These immunogens have been called thymus-independent antigens, and possess in common the structural feature of a repeating antigenic subunit. The above experimental observations have led to the hypothesis that the immune response to immunogens that possess repeating antigenic determinants is independent of the thymus. The repeating antigenic subunit concept cannot totally account for independence from the thymus, however, since the antibody responses to four different multichain synthetic polypeptide immunogens, which are also composed of repeating antigenic determinants, were found to be thymus-dependent (7–9).

It is possible that the four immunogens reported to be thymus-independent possess, in addition to repeating antigenic subunits, another common immunogenic denominator: they are poorly and incompletely metabolized. If thymus-derived helper cells function to concentrate antigen (16), it is conceivable that poorly metabolized immunogens would not require special mechanisms for antigen concentration, and the marrow-derived precursors of antibody-forming cells might generate efficient immune responses without the aid of thymus-derived helper cells. For this purpose, we have systematically investigated a family of four immunogens that possess similar size, shape, amino-acid composition, and probably sequence, but that differ from each other exclusively...

...molecules, is independent of the thymus.

outside and L inside, or exclusively of D-amino acids, the resulting antibody responses were of a similar frequency when marrow cells were injected with or without thymocytes. This observation suggests that the immune response to poorly metabolized immunogens is independent of the thymus.

Since we assumed that the above thymus-independent immunogens are slowly and incompletely metabolized (16, 19), the possibility existed that some regeneration of the thymic component of the immune system could have occurred via repopulation of the irradiated host thymus by the grafted marrow cells, while these poorly-processed immunogens were still effective. In order to exclude this possibility, a cell transfer experiment was performed with thymectomized, irradiated recipients. The results shown in the last line of Table 1, are indistinguishable from the results of the experiment in which the same immunogen with only D-amino acids was injected into thymectomized recipients. Since the addition of thymocytes did not enhance the immune response in thymectomized, irradiated recipients, this experiment indicated that the results obtained were not due to regeneration of the host thymus.

If independence from the thymus is related to slow metabolism of the immunogen, this would be an adequate explanation for the response to the polymer containing D-amino acids exclusively, and for the molecule that has D-amino acids outside and L-amino acids inside (19). However, the polymer that is composed of L-amino acids outside and D-amino acids inside would be expected to have its outside L moiety easily digested, and the immune response towards that portion of the molecule might have been expected to be thymus-dependent. Previous studies have shown that poly-(L-Tyr, L-Glu)-poly(D-Pro-poly(L-Lys) initiated in rabbits the formation of antibodies specific to the poly-(L-Tyr, L-Glu) moiety (18). In contradistinction, SJL/J mice produce, upon injection with this immunogen, mainly antibodies to the polypropyl moiety* (the moiety containing the D-amino acids) but do not produce antibodies to (Tyr, Glu) (the portion of the molecule containing the L-amino acids). This accounts for the thymus-independence of this particular immunogen in SJL/J mice.

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in the optical configuration of the amino acids that compose their various portions. The polypeptide composed exclusively of L-amino acids can be metabolized, whereas two of the polymers, the one composed exclusively of D-amino acids and the other composed of 93% L-amino acids but containing D-amino acids in its outside moiety are slowly metabolized (19). The polypeptide composed of 93% D-amino acids inside, but consisted of L-amino acids on the outside should be degradable only so far as the outside moiety that contained the L-amino acids is concerned.

Results of the cell-transfer experiments summarized in Table 1 demonstrate a correlation between the fact that the immunogen is slowly metabolized and its ability to elicit an antibody response by transferred marrow-cells without the addition of thymocytes. The immunogen composed exclusively of L-amino acids was thymus-dependent, whereas the one containing only D-amino acids did not depend on thymus. Before interpreting the results obtained with the polymers composed of amino acids(L-amino acids outside and n inside, or D-amino acids outside and L inside) it should be emphasized that the antibody responses of SJL mice are directed mainly to the poly(proline) side chains of multicycopoly(Tyr,-Glu)-poly(Pro)-poly(Lys), irrespective of whether these side chains are composed of D- or L-proline. This mouse strain does not generate significant antibody responses to the outside moieties composed of D- or L-tyrosine and glutamic acid. The results obtained with the polymers containing either L-amino acids outside and D inside or D-amino acids outside and L inside indicate that the humoral responses to these two immunogens are independent of the thymus.

The immune response to the copolymer composed of L-amino acids outside and n inside is expected to be independent of the thymus, since the immunopotent region of this macromolecule is made up of poly(D-proline) side chains that are metabolized slowly. One might have expected the antibody response to the immunogen consisting of D-amino acids outside and L inside to be thymus-dependent, since the immunopotent region of this macromolecule is made up of poly(T-proline) side chains. The poly(proline) side chains of this immunogen macromolecule are not easily metabolized, however, since no endopeptidases are known that are capable of splitting peptide bonds between two L-proline residues, and every poly(L-proline) chain in this immunogen is linked to an ε-amino group of lysine on one end and to a peptide composed of D-amino acids on the other imino-terminal end, and thus no significant digestion of this polymer can occur (18, 19). Thus, the immune response specific for the moiety made up of L-amino acids is thymus-independent, probably due to the poor degradation of another part of the immunogen that prevents metabolism of the whole immunogen, including the inside portion of the molecule made up of L-amino acids. This provides an example of one part of an immunogen (which is apparently not involved in the antibody specificity) influencing the need, or lack of need, for thymus-marrow cell cooperation for the production of antibodies specific for another part of the immunogen. The recognition of antigenic determinants has been shown to occur while the immunogen is still intact (21). It is probable, however, that digestion of the immunogenic macromolecule after antigenic recognition is desirable for the production of an efficient antibody response. If this hypothesis is correct, then antigenic recognition of the intact immunogen would be expected to take place before the occurrence of that step in the immune process that deter-

mines whether an antibody response will be thymus-dependent or thymus-independent.

In order to establish whether thymus-dependent and thymus-independent antibody responses could be obtained for two immunopotent regions within the same immunogenic macromolecule, thymus and/or marrow cell transfers were made in DBA/1 mice, which were then immunized with poly(L-Phe,L-Glu)-poly(n-Pro)-poly(n-Lys). In contrast to the immune response to the related polypeptide poly(L-Tyr,-L-Glu)-poly(n-Pro)-poly(n-Lys) discussed above, which elicits antibodies specific for only the proline side chains, two populations of antibodies are produced in DBA/1 mice to the copolymer containing L-phenylalanine, one specific for (L-Phe,L-Glu) and the other specific for the poly(n-proline) side chains*. The results (see Table 2) indicate that the antibody response to the L-amino-acid moiety on the outside is thymus-dependent, whereas the response to the poly(n-proline) side chains on the inside is thymus-independent. This provides an example of an immunogenic macromolecule composed of two immunopotent regions—one of which is thymus-dependent and the other thymus-independent. If the present-held concept of thymus-marrow cell-to-cell interaction involving two separate determinants is considered to be valid for this immunogen, an interesting possibility arises: The antibody response to the moiety composed of D-amino acids on the inside does not require a thymus-derived helper-cell population. Yet this moiety or the entire immunogenic macromolecule probably triggers a thymus-derived cell population in order to provide helper cells for the thymus-dependent antibody response specific for the outside moiety composed of L-amino acids.

Whereas the mouse antibodies formed against the pneumococcal polysaccharide that is independent of the thymus, are of the IgM class exclusively (12), the antibodies produced against the thymus-independent immunogens described here are of the IgM and IgG classes, as concluded from the effect of 2-mercatopetoxyen on hemagglutination titers, and from the fact that both direct and indirect hemolytic plaque-forming cells specific for these immunogens were observed.

Limiting-dilution analyses of immunocompetent-cell populations have been used for estimation of the frequency of precursor cells that elicit the thymus-dependent antibody responses to multichain synthetic polypeptide immunogens composed of L-amino acids (7, 8, 22, 23). A similar approach has been used by Möller and Michael to estimate the frequency of splenic antigen-sensitive cells that react with E. coli lipopolysaccharide antigens, which are independent of the thymus (15). Their results indicate that 10 times more relevant precursor cells were stimulated by this thymus-independent antigen than has been reported for thymus-dependent antigens such as sheep erythrocytes (24-26) and the synthetic polypeptides composed of L-amino acids (22, 23). It was suggested that the frequency of antigen-sensitive cells formed against immunogens that require thymus-marrow cell cooperation reflects the efficiency of the helper mechanism rather than the actual proportion of precursor cells (15). Although careful limiting-dilution studies of the cells that generate responses to the multichain synthetic polypeptides containing D-amino acids have not been made, the frequencies of responses observed for these thymus-independent immunogens were not higher than those obtained for the thymus-dependent copolymers composed of L-amino acids (see Table 1). In fact, a lower proportion of antibody
responses was consistently detected in recipients for the thymus-independent response (either in the presence or absence of thymocytes) than for the thymus-dependent response. The same phenomenon was seen for responses to the thymus-dependent and thymus-independent moieties of poly(L-Phe, L-Glu)-poly(α-Pro)-poly(α-Lys) (see Table 2). Since substances such as poly(A)-poly(U) and methylated bovine serum albumin have been shown to alter the response patterns of cell suspensions detected by limiting dilution (27), the possibility cannot be excluded that endotoxin may have had unspecific stimulating effects on the immune response to E. coli lipopolysaccharide (15).

The results presented in this report indicate that the thymus-independence of immunogens with similar size, shape, and amino-acid composition is related to the optical configuration of some or all of the amino acids that compose their structure. Since thymus-independence of the antibody responses against these five related multichain polypeptides was correlated with the metabolizability of the immunogens or their various moieties, it is likely that for the category of thymus-independent immunogens that possess repeating antigenic determinants, slow metabolism may be a requirement for a steady multipoint binding of antigenic determinants to the lymphocyte, which is in turn needed for the induction of immune response.

The excellent technical assistance of Mrs. Etti Ziv and Mrs. Heidy Zinger is gratefully acknowledged. This investigation was supported in part by agreement 06-035 with the National Institutes of Health, U.S. Public Health Service, Bethesda, Md.