THE FUNCTIONAL DISSECTION OF AN ANTIGEN MOLECULE:
SPECIFICITY OF HUMORAL AND CELLULAR
IMMUNE RESPONSES TO GLUCAGON*

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It has been established over the years that the capacity of an individual to form anti-
bodies to almost any conceivable chemical structure is extremely diverse. That is,
essentially any structural entity can serve as an antigenic determinant or hapten.
However, the “recognition” of a molecule as an immunogen is more restricted. As one
example, poly-γ-α-glutamic acid (PGA)1 of average mol wt 35,000 cannot elicit an
immune response in pure form, but serves as a hapten when complexed to an immuno-
genic carrier (1). Animals which have produced circulating antibody to PGA by virtue
of immunization with PGA-carrier complexes do not exhibit cellular immune reac-
tions, such as delayed hypersensitivity in vivo or lymphocyte stimulation in cell cul-
ture, when challenged with the pure polypeptide (2, 3), and it cannot serve as a carrier
for a conjugated azohapten in normal animals or in animals actively producing anti-
PGA antibody (1). Thus, while there are apparently antibody-forming cells (AFC or
B cell) for determinants on PGA, which is a multivalent hapten on the basis of pre-
cipitation with antibody, it is not recognized as an antigen unless introduced with a
suitable carrier. This is true even when two different determinants are present (PGA–
azohapten conjugates), each of which is an effective hapten (1). A requirement for at
least two different determinants on a molecule to confer immunogenicity has been re-
ported (4). While this may be a necessary condition, it is not a sufficient one if neither
of the determinants fulfills the carrier function required for immunogenicity. Similar
examples have been described (5).

In recent years a need for at least two functionally distinct populations of immuno-
cytes for the induction of humoral immunity has become recognized (6–9). The evi-
dence suggests that one of these is an antigen-reactive cell (ARC or T cell) which does
not secrete antibody but does interact with antigen, presumably via specific surface
receptors. This cell appears to have come under the influence of the thymus at some

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1 Abbreviations used in this paper: AFC, antibody-forming cells; ARC, antigen-reactive
cells; C, carboxy-terminal undeca- and dodecapeptide; NM, amino-terminal heptadecapep-
tide; PGA, polyglutamic acid; TPCK, 1-tosylamide-2 phenylethyl-chloromethyl ketone.
point in its history and is needed for the initiation of cellular immune phenomena, the elicitation of which distinguishes immunogens from haptens. Evidence for these relationships derives from human disease (diGeorge's syndrome) as well as from experimental work with animals.

Consistent with these findings is the supposition that nonimmunogenicity of haptens is due to the absence of a structural determinant on the molecule which can interact above a threshold thermodynamic level with receptors on one or more clones of ARC. It follows from this that every immunogenic molecule has at least one such determinant. Using small, structurally defined immunogens, it should be possible to dissect the immunogenic or recognition determinant(s) from haptenic determinants which dictate the specificity of serum antibody. This has been accomplished using glucagon, a polypeptide of 29 amino acids. A brief report of the earliest findings has been published (10).

Materials and Methods

Antigen.—Crystalline bovine glucagon was a gift from Dr. W. Bromer. Additional quantities were purchased from Mann Research Laboratories Inc., New York. The preparation of tryptic fragments of glucagon was carried out as described (11). An 0.085% solution of glucagon was incubated with 0.005% trypsin (1-tosylamide-2% phenylethyl-chloromethyl ketone [TPCK]-treated) in 0.05 M sodium citrate containing 1 mg/ml of CaCl₂ at 25°C, pH 7.8, for 2.5 hr with gentle shaking. The digest was then adjusted to pH 3.0 with HCl, heated to 90°C for 2 min, and lyophilized. The resulting three fragments (N, M, and C, Fig. 1) and free arginine were separable by high-voltage electrophoresis on paper (pH 3.5, 3000 v for 1 hr). They were initially identified by differential staining for arginine, tyrosine-histidine, and tryptophan, and their identity was later confirmed by amino acid analysis using a Beckman model 120 C analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

The fragments were prepared by washing the lyophilized digest with 0.1 N acetic acid. The acid soluble fraction contained fragments N and M, which were resolved on Sephadex G-25. M was desalted by passage through Sephadex G-10. The acid insoluble precipitate was vacuum-dried and the C fragment was purified by preparatory high-voltage electrophoresis on paper at pH 3.5; it was eluted with 1 N ammonia.

To obtain the NM fragment (Fig. 1), lysine was blocked using citraconic anhydride (2-methyl-maleic anhydride) as previously described for insulin (12). For every 15 mg of glucagon, 100 mg of citraconic anhydride was added and the mixture was stirred at room temperature while maintaining the pH at 8.0 by addition of NaOH. Base uptake ceased after about 10 min and the preparation was desalted by filtration through Sephadex G-25 in 0.5% NH₄HCO₃. Tryptic digestion of the blocked polypeptide was carried out as described above. To remove the blocking group, the lyophilized digest was acidified and left at room temperature overnight. The acid soluble fraction was chromatographed on Sephadex G-25 in 0.1 N acetic acid and the NM peptide was obtained in pure form.

Animals and Immunization.—Randomly bred guinea pigs of both sexes weighing approximately 600 g were immunized with 0.1–1.0 mg of glucagon emulsified in complete Freund's adjuvant and distributed among all four footpads. Animals were bled 2 wk later.

Radioimmunoassay for Anti-Glucagon Antibody.—Glucagon was radiolabeled with ¹²₅I as described for the iodination of insulin (13). All glassware exposed to the isotope was siliconized. The iodinated product was purified by passage through Sephadex G-25 and had a specific activity of 0.33 μCi/μg.
FUNCTIONAL DISSECTION OF AN ANTIGEN MOLECULE

To assay for antibody, a dextran-coated charcoal radioimmunoassay described for insulin (14) was adopted. Coated charcoal was prepared by mixing equal volumes of 5% charcoal (Decolorizing Carbon, Norit, Eastman organic Chemicals, Rochester, N.Y.) and 0.5% Dextran 500 (Pharmacia Fine Chemicals, Piscataway, N.J.) in 0.1 M phosphate buffer, pH 7.4. Assays were performed in siliconized glass tubes. 50 μl of 121I-glucagon containing approximately 2000 cpm were added to varying quantities of serum or γ-globulin fractions of serum. Volumes were brought to 0.5 ml with 0.1 M phosphate buffer, pH 7.4, and the tubes were incubated at 4°C for 1 hr. For assays of the inhibition of binding of radiolabeled glucagon, inhibitors were added to tubes containing a quantity of serum which bound 50-60% of the counts and incubation at 4°C was extended to 18-24 hr. After incubation, the tubes were transferred into an ice bath and 0.5 ml of a suspension of cold coated charcoal was added to each tube. The tubes were mixed and centrifuged for 10 min at 1500 rpm. A sample of each supernatant was transferred into fresh tubes and the tubes were counted in a Packard Auto-Gamma Spectrometer (Packard Instrument Company, Downers Grove, Ill.). The fraction of total radioactivity in the supernatant was taken as the measure of glucagon bound by antibody. Assays with pre-immune sera established the baseline. In no instance did such a serum bind more than 5% of the radioactivity.

Skin Tests. 40-100 μg of antigen in 0.1 ml of phosphate-buffered saline, pH 7.4, were injected intradermally into a shaved area of the flank. Reactions were recorded 2 and 24 hr after challenge. An area of induration 5 mm or larger 24 hr after challenge was considered a positive delayed hypersensitivity reaction.

Capillary Migration of Peritoneal Exudate Cells (15).—Peritoneal exudates were induced by intraperitoneal injection of 20 ml of light mineral oil. 3 days later the cells were harvested by washing the peritoneal cavity with 200 ml of Hanks' solution. Packed cells were suspended in Spinner's medium, 7.5% by volume, and capillary tubes were filled with the cell suspension. The tubes were sealed and centrifuged in the cold for 5 min at 900 rpm. They were cut at the cell-fluid interface and placed in Mackaness-type culture chambers, two tubes per chamber; chambers were prepared in duplicate or triplicate. The chambers were filled with Spinner's medium containing 100 units of penicillin and 100 units of streptomycin/ml, 1% L-glutamine, 15% normal guinea pig serum, and various concentrations of the test antigen. The chambers were then sealed and incubated for 24-48 hr at 37°C. Migration patterns were projected and traced, and the areas were measured using a planimeter. The areas of migration in duplicate test chambers (four tubes) were averaged, and the results were expressed as per cent inhibition of migration in chambers containing antigen based on comparison with the migration of cells in chambers containing no antigen.

Lymphocyte Stimulation (16).—4-5 × 10⁶ lymph node cells were cultured in 2 ml of Eagle's minimal essential medium containing 100 units each of penicillin and streptomycin/ml, 1% L-glutamine, and 5% calf serum. Antigen was added in 0.1 ml of saline and the cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air for 2 days, at the end of which period 0.05 μCi of 14C-methyl-thymidine (New England Nuclear Corp., Boston, Mass.; specific activity 24.5 mCi/mM) was added to each culture tube. After an additional 24 hr incubation, the cells were harvested in the cold by serially suspending them once in saline, twice in 5% trichloroacetic acid, and once in absolute methanol. The dried residue was dissolved by incubation in 1 ml of hyamine at 56°C for 1 hr followed by 3-24 hr at 37°C, transferred to counting vials, and prepared for scintillation counting by addition of 12 ml of Omnifluor (New England Nuclear Corp.) in toluene (4 g of Omnifluor in 1 liter toluene). Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer. The variation between replicate cultures seldom exceeded ±5%.

Peptide Synthesis. A series of peptides corresponding to the amino-terminal portion of glucagon were synthesized using the solid-phase technique (17). The series was initiated at
residue 16 (serine) and extended back to residue 2 (serine). The peptides were assayed for their capacity to block the binding of radiolabeled glucagon by antiserum. A detailed account of their preparation, purification, and characterization has been published (18).

Another series of peptides corresponding to the carboxy-terminal sequence of glucagon were synthesized using a somewhat modified approach. The presence of tryptophan and methionine in this sequence necessitated the use of protective reagents such as anisole and 1,2-ethane di-thiol to minimize oxidation during synthesis and in the final cleavage of the product from the resin. Two side reactions were noted. After addition of aspartic acid, cyclization of the aspartyl residue occurred to some extent, as previously observed (19). A previously unreported rearrangement, N to O acyl migration involving threonine was observed beginning at the dipeptide stage. The products became very poorly soluble after the pentapeptide stage precluding analysis for impurities by high-voltage electrophoresis. A number of purification techniques were attempted. The best results were obtained by repeated passage through Sephadex G-15 in 1 N NH₄OH. The purified products were characterized by amino acid analysis. They were assayed for capacity to stimulate lymphocytes from glucagon-sensitized animals.

RESULTS

Characterization of Peptides.—Amino acid analyses of glucagon and its purified NM and C tryptic fragments are shown in Table I. By analyzing

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TABLE I

| Amino Acid | Glucagon | NM | C |
|------------|----------|----|----|
|            | Expected | Found | Expected | Found | Expected | Found |
| Lysine     | 1.00     | 1.00 | 1.00 | 1.00 | 0.00  | 0.01  |
| Histidine  | 1.00     | 0.91 | 1.00 | 0.95 | 0.00  | 0.01  |
| Arginine   | 2.00     | 2.29 | 1.00 | 1.18 | 0.00  | 0.77  |
| Aspartic acid | 4.00   | 4.11 | 2.00 | 2.00 | 2.00  | 2.04  |
| Threonine  | 3.00     | 2.25 | 2.00 | 1.46 | 1.00  | 0.96  |
| Serine     | 4.00     | 3.68 | 4.00 | 3.95 | 0.00  | 0.00  |
| Glutamic acid | 3.00 | 3.14 | 1.00 | 1.13 | 2.00  | 2.04  |
| Proline    | 0.00     | 0.00 | 0.00 | 0.00 | 0.00  | 0.00  |
| Glycine    | 1.00     | 0.86 | 1.00 | 1.00 | 0.00  | 0.00  |
| Alanine    | 1.00     | 1.09 | 0.00 | 0.00 | 1.00  | 1.00  |
| Half-cystine | 0.00  | 0.00 | 0.00 | 0.00 | 0.00  | 0.00  |
| Valine     | 1.00     | 1.00 | 0.00 | 0.00 | 1.00  | 1.05  |
| Methionine | 1.00     | 1.00 | 0.00 | 0.00 | 1.00  | 0.96  |
| Isoleucine | 0.00     | 0.00 | 0.00 | 0.00 | 0.00  | 0.00  |
| Leucine    | 2.00     | 2.04 | 1.00 | 0.95 | 1.00  | 1.00  |
| Tyrosine   | 2.00     | 1.98 | 2.00 | 1.95 | 0.00  | 0.01  |
| Phenylalanine | 2.00 | 2.09 | 1.00 | 1.00 | 1.00  | 0.96  |

* M/M* = moles per mole.
† Ratios normalized to this amino acid.

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varying quantities of the fragments, it was established that they had a degree of purity of at least 99%. Amino acids which would represent contamination by either intact glucagon or the complementary fragment were never found in significant quantity, even when the columns were overloaded with the peptide hydrolysates.

The C fragment proved to be a mixture of undeca- and dodecapeptides, due to incomplete cleavage of the second of two consecutive arginine residues at position 18 (Fig. 1). This was established by recovery of less than the quantity of free arginine expected on the basis of complete cleavage, and by the appearance of arginine in fractional molar quantities in the analysis of C (Table I). The two C peptides were surprisingly resolved by chromatography on
Sephadex G-15 in 1 N ammonia. Only the second peak contained arginine, indicating that resolution was due to adsorption of the larger peptide to the gel.

Specificity of Humoral Antibody.—All guinea pigs immunized with glucagon responded with humoral antibody detected by the binding of $^{125}$I-glucagon. 50 µl of antiserum bound from 50 to 80% of the 2000 cpm used for each assay, whereas preimmune sera consistently bound less than 5%. The tryptic fragments of glucagon inhibited this binding in the order NM > C > N > M. Curves for a representative serum are shown in Fig. 2. While the increments between the ligands varied from one serum to another, the order of effectiveness did not. M peptide was totally ineffective in all assays, and the molar ratio C:NM required for a given level of inhibition was never less than 5:1 and frequently much greater.

When the fragments were added in pairs to assess their combined inhibitory effect, it was found that the combination of N and C was additive, indicating that the two fragments reacted with distinct antibody populations. NM and C also produced an additive effect, raising the level of inhibition to that given by intact glucagon. However, since in some antisera the inhibition given by NM and C separately added up to more than 100%, some overlap appears likely. These results show that the antibodies produced in response to glucagon are heterogeneous in specificity, but the major antigenic determinant in all animals tested is carried by the NM fragment (residues 1-17).

The structural requirements of the antigenic determinant in NM for binding with antibody were assessed by assaying a series of synthetic peptides for inhibitory capacity. The series was initiated at residue 16 because of difficulties

![Graph](image-url)
caused by arginine (residue 17) during synthesis. The smallest peptide with demonstrable activity was a dodecapeptide encompassing residues 5–16 of glucagon (Fig. 3). Larger peptides were somewhat more effective but did not yield a consistent gradient of increasing activity with size, and none proved as active as native NM. It is of interest that X-ray diffraction studies indicate that glucagon is about 75% helical, with two sections of helix. The model which best fits the data and permits the most stable placement of hydrophobic residues predicts a helical segment between residues 5–16 and another between residues 17–28 (20). In conjunction with this model, the relative ineffectiveness of the N peptide and the initial appearance of activity with synthetic peptide 5–16 suggest that reactivity with antibody may depend strongly on the conformation of the amino-terminal portion of glucagon.

**Specificity of Cellular Immunity.**—Cellular immune activities of glucagon and its fragments were assayed by their ability to elicit delayed cutaneous hypersensitivity reactions, to inhibit capillary migration of peritoneal exudate cells, and to stimulate the synthesis of deoxyribonucleic acid (DNA) by lymphocytes in vitro. In addition, limited investigations of the immunogenicity of NM and C peptides were conducted.

(a) **Delayed hypersensitivity:** Animals were skin tested with up to 100 μg of
glucagon and the fragments. All animals tested gave positive delayed reactions with glucagon and C, and somewhat weaker though unambiguous reactions with NM (Table II). N was only marginally active, with 1 of 6 animals giving a mildly positive reaction, and M was totally inactive. None of the preparations gave positive reactions when tested in normal guinea pigs, and none of the immune guinea pigs showed a positive reaction with any of the antigens 2 hr after injection.

(b) Inhibition of capillary migration: To confirm the results of delayed skin reactions, inhibition of migration of peritoneal cells from capillary tubes was performed. The results of the two tests correlated perfectly (Table II). Glucagon, C, and NM, at concentrations of 50-100 μg/ml of culture medium,

| Test antigen | Average delayed skin reaction | Inhibition of cell migration | Lymphocyte stimulation |
|--------------|-----------------------------|----------------------------|-----------------------|
|              | mm                          | %                          | Range | Mean |
| Glucagon     | 14 (20)§                    | 65 (8)                     | 2.3-26.0 | 10.9 (13) |
| N            | 3 (6)                       | 21 (3)                     | 0.7-1.1 | 0.8 (6) |
| M            | 0 (6)                       | 0 (3)                      | 2.7-20.0 | 8.8 (13) |
| NM           | 8 (6)                       | 55 (3)                     |        |      |
| C            | 12 (6)                      | 75 (3)                     | 2.7-20.0 | 8.8 (13) |

* The figures were derived from the following equation:

\[
\frac{1 - \text{migration of cells in chambers containing antigen}}{\text{migration of cells in chambers without antigen}} \times 100
\]

† Ratio of 3H-thymidine incorporation in cultures with antigen present relative to cultures without antigen from the same animal.

§ Parentheses indicate number of animals tested.

inhibited migration strongly, whereas N exhibited only a mild inhibitory effect and M was again inactive. None of the preparations was inhibitory when tested with peritoneal cells from normal animals.

(c) Lymphocyte stimulation in vitro: Glucagon and C were highly active and almost equivalent in their capacities to stimulate lymph node cells from immune animals to synthesize DNA (Tables II and III). But whereas glucagon and C stimulated as much as 26-fold and 20-fold respectively, NM did not stimulate significantly above background over the concentration range tested.

This pattern was very consistent (Table III). Moreover, with cells from two animals which were stimulated somewhat better by glucagon than by C, the addition of equimolar quantities of NM to C did not enhance its transforming activity (Table III). Sera from all these animals showed the pattern of speci-
TABLE III
In Vitro $^{14}C$-Thymidine Incorporation in Response to Glucagon Peptides in Lymph Node Cell Cultures Derived from Glucagon-Sensitized Guinea Pigs

| Animal | Glucagon (0.15–28.00) ‡ | Maximum stimulation* given by (0.25–50.00) (3–33) |
|--------|--------------------------|--------------------------------------------------|
| NM     | C NM + C                 |                                                  |
| 1      | 15.0                     | 16.0                                             |
| 2      | 14.1                     | 12.5                                             |
| 3      | 12.0                     | 8.6                                              |
| 4      | 2.3                      | 4.1                                              |
| 5      | 17.8                     | 18.0                                             |
| 6      | 7.2                      | 5.9                                              |
| 7      | 11.0                     | 9.2                                              |
| 8      | 6.2                      | 1.1                                              |
| 9      | 26.0                     | 1.1                                              |
| 10     | 6.4                      | 0.8                                              |
| 11     | 13.2                     | 1.1                                              |
| 12     | 5.7                      | 0.9                                              |
| 13     | 4.4                      | 0.7                                              |

* Ratio of $^{14}C$-thymidine incorporation in cultures with antigen present relative to cultures without antigen from the same animal.
‡ Concentration range used, in millimicromoles per milliliter of culture medium.

TABLE IV
Stimulation of Lymph Node Cell Cultures from Glucagon-Sensitized Animals by Natural and Synthetic Peptides

| Animal | Glucagon | Synthetic peptides |
|--------|----------|--------------------|
|        | C        | C  ‡ | C § | dodeca | undeca | decap |
| 1      | 12.0     | 8.6  | 6.8 | 1.6    | 3.5    | 1.0   |
| 2      | 15.0     | 16.0 | 1.0 | 0.8    | 9.0    | 1.3   |
| 3      | 14.0     | 12.5 | 3.8 | 3.8    |        |       |
| 4      | 17.8     | 18.0 |     | 2.1    |        |       |
| 5      | 11.0     | 9.2  | 7.1 | 1.1    | 1.1    |       |
| 6      | 15.0     | 12.7 | 10.2| 10.5   | 4.3    | 4.3   |

* Ratio of $^{14}C$-thymidine incorporation in cultures with antigen present relative to cultures without antigen from the same animal.
‡ Natural carboxy-terminal undecapeptide of glucagon.
§ Natural carboxy-terminal dodecapeptide of glucagon.

Functionality described earlier. NM was always a much more effective inhibitor than C of the binding of radiolabeled glucagon by antiserum.

Synthetic peptides simulating the C fragment confirmed the transforming activity of this portion of the glucagon molecule (Table IV). The synthetic
peptides had to be extensively purified by repeated passage through Sephadex 
G-15 because of a toxic effect on lymphoid cells, possibly due to residual traces 
of anisole and 1,2-ethane dithiol in the preparations. Even the purest prepara-
tions were less active than native C, despite amino acid analytical data in good 
agreement with theoretical ratios (Table V). Peptides smaller than the unde-
capeptide were inactive with cells which were strongly stimulated by native 
C as well as by the synthetic undecapeptide. Surprisingly, the synthetic 
dodecapeptide was also much less active than the undecapeptide in all save one 
instance. This was confirmed by assaying the undeca- and dodecapeptides of 
native C, which were resolved by passage through Sephadex G-15. The fraction 
which analyzed for arginine and is therefore the dodecapeptide is designated 

| Amino Acid | Undecapeptide | Dodecapeptide |
|------------|---------------|---------------|
| Aspartic acid | 2.00 | 2.16 | 2.00 | 2.13 |
| Threonine | 1.00 | 1.07 | 1.00 | 1.03 |
| Glutamic acid | 2.00 | 1.87 | 2.00 | 1.74 |
| Alanine | 1.00 | 0.99 | 1.00 | 0.97 |
| Valine | 1.00 | 0.98 | 1.00 | 0.92 |
| Methionine | 1.00 | 0.94 | 1.00 | 0.92 |
| Leucine | 1.00 | 1.00§ | 1.00 | 1.00§ |
| Phenylalanine | 1.00 | 0.98 | 1.00 | 0.93 |

* Tryptophan and nitroarginine are not included because they are largely destroyed 
during hydrolysis.
‡ M/M = moles per mole.
§ Ratios normalized to this amino acid.

C₂ in Table IV; the arginine-less undecapeptide is designated C₁. It can be 
seen that the relative effectiveness of C₁ and C₂ correlated with that of the 
synthetic peptides and varied from one animal to another. The variation 
ranged from one individual in which C₁ stimulated 16-fold, while C₂ was 
virtually inactive, to another in which the two were indistinguishable. The 
addition of arginine, which is the only basic residue in this sequence, to the 
undecapeptide drastically reduced its transforming activity in most cases, 
despite the presence of arginine at that position in the native sequence. None 
of the native or synthetic peptides had a stimulatory effect on cells from normal 
animals.

Immunogenicity of NM and C Peptides.—Three guinea pigs in each group 
were immunized with 0.4 mg of natural NM or C in complete Freund’s adju-
vant, distributed equally between the four footpads. They were bled and skin
tested during the 3rd wk after immunization. Using glucagon as the antigen, this regimen invariably results in skin sensitization and circulating antibody. None of these animals gave delayed hypersensitivity skin reactions when tested with up to 100 μg of glucagon and the peptides used for immunization, nor could glucagon-binding antibodies be demonstrated in any of the sera. Thus, neither of the peptides, if immunogenic at all, is as strongly immunogenic as the parent molecule.

DISCUSSION

The central finding of this study is a striking dissociation in virtually all the guinea pigs assayed between the specificity of circulating anti-glucagon antibody and the specificity of immunocytes which are transformed by antigen in culture. The amino-terminal heptadecapeptide (NM) was a far more effective inhibitor of the binding of glucagon by antibody than was the carboxy-terminal undec- or dodecapeptide (C). On the other hand, NM was totally unable to trigger lymphoid cells to synthesize DNA, whereas C was almost as active, if not as active, as intact glucagon in this capacity. Although amino acid analyses of NM and C indicated a very high degree of purity, the inherent transforming activity of C was unambiguously substantiated with synthetic peptides.

The use of synthetic C peptides demonstrated that residue 19, alanine, is essential for transforming activity, since the carboxy-terminal decapetide was inactive whereas the undecapetide was highly active. However, it is not known how much of the sequence carboxy-terminal to residue 19 is required for this activity. A very stringent structural requirement for cellular stimulation was indicated by the finding that the natural and synthetic dodecapep- tides were markedly less active than their undecapeptide counterparts with cells from most of the animals tested. Nevertheless, the pattern of reactivity varied from animal to animal, and cells from one guinea pig did not distinguish between the undeca- and dodecapeptides (Table V). This kind of variation is not unlike that seen in the specificities of populations of antibody molecules from different animals. The loss of activity in the dodecapeptide could be due to the charge introduced by arginine, the only basic residue in the sequence, or to an altered conformation of the peptide. It would be of interest to determine at which point activity is recovered upon lengthening the peptide; restoration must occur since the parent molecule is fully active. Studies to answer this question are in progress.

These findings are very easily reconciled with the now widely accepted two-cell mechanism of immune induction (6–9). The antigen-reactive cell, or T cell, must recognize a molecule in order for it to be immunogenic. Subsequently, there is an as yet undefined interaction between the T cell and an antibody-producing cell precursor, or B cell, presumably but not necessarily through the intermediation of the antigen molecule, which causes the B cell to differentiate
into a mature antibody-producing cell. Although interaction between the cells and antigen appears to be specific, the parts of an antigen molecule with which the two cells interact may be mutually exclusive. In the case of glucagon, we propose that the major immunogenic or recognition determinant for T cells is carried by the C peptide, whereas the major haptenic determinant for B cells is carried by the NM fragment. Obviously, this distinction need not be absolute. The antibodies produced against glucagon are heterogeneous in specificity and a minor population in a considerable fraction of the animals tested showed specificity for the C peptide. Similarly, there may be T cells with specificity for an amino-terminal determinant of glucagon, but they may go undetected either because the assay method is too insensitive or because they do not react with the NM fragment. It does not appear likely that such a population of T cells could be substantial, since C and glucagon showed very similar stimulatory activities with cells from most of the animals (Table III).

The immunogenic determinant of a molecule can be regarded as fulfilling the carrier function of a hapten-carrier conjugate and might be expected to be immunogenic itself, at least to the extent of provoking cellular immunity. The limited experimental testing of the immunogenicity of the tryptic peptides reported here was negative, indicating that C is at best a weaker immunogen than glucagon. However, more extensive tests over a broader dosage range of antigen will be necessary before firm conclusions can be drawn.

Several investigators have demonstrated a correlation between delayed cutaneous hypersensitivity and inhibition of migration of cells from capillary tubes (15, 21–23). The findings with glucagon peptides are consistent with those of others inasmuch as each peptide which elicited a skin reaction also inhibited cell migration, and their effectiveness in the two assays showed a roughly quantitative correlation (Table II). However, we failed to find a correlation between skin reactivity or inhibitory capacity on the one hand, and the ability to transform cells in vitro on the other, since the NM peptide was decidedly active in the two former assays but exerted no palpable effect on DNA synthesis (Table III). This contrasts with several reports (21, 24), but is in agreement with others which demonstrated a dissociation between these activities (23, 25). It is unclear whether the activities represent different expressions by the same cells or expressions by different populations of cells. In either case, a particular ligand could conceivably trigger the release of migration inhibitory factor without activating DNA synthesis. To our knowledge, there are no known instances of the reverse, antigen-specific cell stimulation in the absence of in vivo or in vitro delayed hypersensitivity.

SUMMARY

Bovine glucagon, a polypeptide of 29 amino acids, was immunogenic in guinea pigs. The immunologic determinants of glucagon were investigated using
isolated tryptic peptides of the hormone. Antibodies from virtually all of more than two dozen animals had specificity primarily for the amino-terminal heptadecapeptide (NM) and showed little or no binding with the carboxy-terminal undeca- and dodecapeptides (C). The smallest synthetic peptide of a series initiated at residue 16 which measurably bound antibody comprised residues 5–16 of glucagon.

In cellular immune assays, both NM and C elicited delayed cutaneous reactions and inhibited the migration of peritoneal cells from immune animals. However, only intact glucagon and its C fragment stimulated lymphoid cells to synthesize DNA. While glucagon was somewhat more active than C, the addition of NM to C did not enhance its transforming activity. The smallest synthetic carboxy-terminal peptide with discernible transforming activity comprised residues 19–29 of glucagon. In both native and synthetic C peptide preparations, the undecapeptide was generally more active than the dodecapeptide, although cells from different animals gave different response patterns. The difference between the two is the presence of arginine at the amino-terminus of the peptide chain. Thus, the recognition specificity of populations of antigen-reactive cells from different animals displays a variation which is at least superficially analogous to that of populations of antibody molecules.

In limited experiments using NM and C peptides as immunogens, neither gave rise to delayed hypersensitivity or to glucagon-binding circulating antibody, following a regimen which invariably provoked these responses when glucagon itself served as the immunogen.

These results indicate that glucagon was cleaved by trypsin along functional lines into two parts, one of which housed the major antigenic determinant and the other of which carried the major immunogenic determinant, and they are highly compatible with a two-cell mechanism of immune induction. An apparent dissociation between the capacity to provoke delayed hypersensitivity reactions and to transform antigen-reactive cells in culture was observed.

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BIBLIOGRAPHY

1. Goodman, J. W., and D. E. Nitecki. 1967. Studies on the relation of a prior immune response to immunogenicity. *Immunology.* **13:**577.
2. Roelants, G. E., G. Senyk, and J. W. Goodman. 1969. Immunochemical studies on the polyγ-D-glutamyl capsule of *Bacillus anthracis.* V. The in vivo fate and distribution in rabbits of the polypeptide in immunogenic and non-immunogenic forms. *Israel J. Med. Sci.* **5:**196.
3. Roelants, G. E., and J. W. Goodman. 1970. Tolerance induction by an apparently non-immunogenic molecule. *Nature (London).* **227:**175.
4. Rajewsky, K., V. Schirrmacher, S. Nase, and N. K. Jerne. 1969. The requirement
of more than one antigenic determinant for immunogenicity. *J. Exp. Med.* **129**:1131.

5. McDevitt, H. O., and B. Benacerraf. 1970. Genetic control of specific immune responses. *Adv. Immunol.* **11**:31.

6. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.

7. Mosier, D. E. 1967. A requirement for two cell types for antibody formation in vitro. *Science (Washington).* **158**:1573.

8. Haskill, J. S., P. Byrt, and J. Marbrook. 1970. In vitro and in vivo studies of the immune response to sheep erythrocytes using partially purified cell preparations. *J. Exp. Med.* **131**:461.

9. Shearer, G. M., and G. Cudkowicz. 1969. Distinct events in the immune response elicited by transferred marrow and thymus cells. I. Antigen requirements and proliferation of thymic antigen-reactive cells. *J. Exp. Med.* **130**:1243.

10. Senyk, G., D. Nitecki, and J. W. Goodman. 1971. Immunogenicity of glucagon: determinants responsible for antibody binding and lymphocyte stimulation. *Science (Washington).* **171**:407.

11. Bromer, W. W., A. Staub, L. G. Sinn, and O. K. Behrens. 1957. The amino acid sequence of glucagon. III. The hydrolysis of glucagon by trypsin. *J. Am. Chem. Soc.* **79**:2801.

12. Dixon, H. B. F., and R. N. Perham. 1968. Reversible blocking of amino groups with citraconic anhydride. *Biochem. J.* **109**:312.

13. Burrows, B. A., T. Peters, and F. C. Lowell. 1957. Physical binding of insulin by gamma globulins of insulin-resistant subjects. *J. Clin. Invest.* **36**:393.

14. Herbert, V., K.-S. Lan, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay for insulin. *J. Clin. Endocrinol. Metab.* **25**:1375.

15. David, J. R., S. Al-Askari, H. S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro. I. The specificity of inhibition of cell migration by antigens *J. Immunol.* **93**:264.

16. Dutton, R. W., and J. D. Eady. 1964. An in vitro system for the study of the mechanism of antigenic stimulation in the secondary response. *Immunology.* **7**:40.

17. Merrifield, R. B. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85**:2149.

18. Nitecki, D. E., G. Senyk, E. B. Williams, and J. W. Goodman. 1971. Immunologically active peptides of glucagon. Intra-Science Research Foundation. Symp. on Chemistry of Peptides, Santa Monica, Calif. In press.

19. Ondetti, M. A., A. Deer, L. T. Sheehan, L. Pluscec, and O. Kocy. 1968. Side reactions in the synthesis of peptides containing the aspartylglycyl sequence. *Biochemistry.* **7**:4069.

20. Schiffer, M., and A. B. Emundson. 1970. Prediction of alpha helices in glucagon. *Biophys. J.* **10**:293.

21. David, J. R., and S. F. Schlossman. 1968. Immunochemical studies on the specificity of cellular hypersensitivity. The in vitro inhibition of peritoneal exudate cell migration by chemically defined antigens. *J. Exp. Med.* **128**:1451.
22. Godfrey, H. P., H. Baer, and S. D. Chaparas. 1969. Inhibition of macrophage migration by a skin-reactive polysaccharide from BCG culture filtrates. *J. Immunol.* 102:1466.

23. Spitler, L., E. Benjamini, J. D. Young, H. Kaplan, and H. H. Fudenberg. 1970. Studies on the immune response to a characterized antigenic determinant of the tobacco mosaic virus protein. *J. Exp. Med.* 131:133.

24. Mills, J. A. 1966. The immunologic significance of antigen induced lymphocyte transformation in vitro. *J. Immunol.* 97:239.

25. Chaparas, S. D., D. E. Thor, H. P. Godfrey, H. Baer, and S. R. Hedrick. 1970. Tuberculin-active carbohydrate that induces inhibition of macrophage migration but not lymphocyte transformation. *Science (Washington).* 170:637.