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Research Article

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In Vitro Lymphocyte Proliferation Response to Therapeutic Insulin Components

EVIDENCE FOR GENETIC CONTROL BY THE HUMAN MAJOR HISTOCOMPATIBILITY COMPLEX

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ABSTRACT Genes in the major histocompatibility complex of mice and guinea pigs control immunologic responsiveness to insulins from other animal species. In order to determine if similar genetic control exists in man, we have examined lymphocyte proliferation responses to components of therapeutic insulins by employing lymphocytes from diabetic patients that receive insulin.

Distinct groups of individuals demonstrated positive lymphocyte proliferative responses to beef insulin, beef and pork insulin, beef proinsulin, pork proinsulin, and protamine. Lymphocytes from the patient population were typed for the HLA-A, B, C, and DR antigens. An increased frequency of certain HLA antigens was found in those individuals that responded to the following therapeutic insulin components: beef, HLA-DR4; beef and pork, HLA-DR3; beef proinsulin, HLA-BW4, CW2, CW5, DR2, and DR5; protamine, HLA-CW3, CW5, and DR7. The results demonstrate that the human immune system recognized the structural differences between human and beef and/or pork insulin. These differences are two amino acids in the A chain, alpha loop, of beef insulin and the single terminal amino acid, alanine, which is common to pork and beef insulins. Positive responses to both beef proinsulin and pork proinsulin demonstrated the capability of restricted recognition of more complex proteins represented by the C-peptide in these insulin preparations. Lymphocyte proliferative responses to protamine were also restricted, which suggests a genetic control to this antigen. The association of these responses with HLA alloantigens strongly suggests that genes within the human major histocompatibility complex control recognition and lymphocyte response to therapeutic insulin components.

INTRODUCTION

The immune response to protein antigens in experimental animals is controlled by genetic loci that are associated with the major histocompatibility complex (1). These studies have largely been performed in inbred strains of mice or guinea pigs, where it has been clearly demonstrated that immune response (Ir)[1] genes map within the major histocompatibility complex (MHC) of the respective species. Insulins are one of a number of immunogens that have been used to map Ir genes. Using a variety of insulin analogues, MHC

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[1] *Abbreviations used in this paper:* C-peptide, connecting peptide; Ir genes, immune response genes; JOD, juvenile onset diabetes; MHC, major histocompatibility complex.

genes in both mice (2, 3) and guinea pigs (4, 5) have been demonstrated to control the immune response to restricted differences in amino acid composition of the A and B chains of the insulin molecule. Similarly, some mice have been shown to develop an immune response to proinsulin, but not to the insulin itself (6).

In light of the probability that immune response genes also operate in man (7, 8), we undertook a study to examine the possibility that insulin-receiving diabetic patients had restricted lymphocyte proliferative response to components present in therapeutic insulins. These included beef and pork insulin, beef and pork proinsulin, and protamine, which is an agent added to retard the absorption of insulin. This study was predicated on the knowledge that restricted immune responses are observed in mice and guinea pigs that are actively immunized with the insulins used for replacement therapy in man, and we reasoned that similar genetic control of these insulins should be present in man.

The results reported herein demonstrate that lymphocytes from individuals that receive insulin have restricted in vitro lymphocyte proliferative responses to beef and pork insulins, the connecting peptide (C-peptide) of these insulins, and protamine. The association of these responses with increased and/or decreased HLA alloantigen frequencies suggests MHC-linked genetic influence on the immune response of humans to these antigens.

METHODS

Patient population. A total of 137 patients were included in this study. 29 of the patients were Black and 108 were Caucasians. All patients under the protocol were admitted to the Clinical Center at the National Institutes of Health and were withdrawn from exogenous insulin for at least 48–72 h before blood samples were obtained. The mean age of onset of the diabetes in Caucasians was 34.2 yr, and in Blacks, it was 37.8 yr. The mean age at the time of the study was 52.1 in the Caucasians and 49.4 yr in the Black patient populations. The clinical features of 117 of these patients who manifest allergic reactions to insulin or components of therapeutic insulin is reviewed elsewhere (9, 10).

Lymphocyte proliferation. Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque gradient separation and washed four times. Cell concentration was adjusted to 1×10^5 cells/200 μl in RPMI 1640 (containing 10% filtered heat-inactivated AB negative human serum, 10 mM Hepes buffer, 100 U/ml penicillin, and 10 mg/ml gentamycin) and cultured in round-bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) for 5 d at 37°C in 5% CO_2. Cultures were then pulsed for 18 h with 1 μCi/well of [^{3}H]thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) and harvested on glass fiber filter paper by the use of a semi-automated microharvesting device (ADAPS Co., Dedham, MA). [^{3}H]thymidine incorporation was measured by liquid scintillation spectroscopy. 1, 10, and 100 μg of the following antigens were tested in triplicate for their ability to produce lymphocyte stimulation: beef insulin, pork insulin, beef proinsulin, pork proinsulin, and protamine. The insulins and proinsulins were gifts from Dr. Ronald Chance of Eli Lilly and Co. (Indianapolis, IN). Insulin preps had <10 parts per million of their respective proinsulins. Proinsulins contained 1% of respective insulins. Each of the patients was tested on two separate occasions with these antigens. No significant differences in ratios of response or Δ cpm were seen in the lymphocyte stimulation studies that were performed on these two occasions. Ratios of response were calculated using base-line [^{3}H]thymidine incorporation (mean counts per minute) in the triplicate cultures (no antigen) and the mean counts per minute in triplicate cultures at the antigen concentration that gave maximal stimulation. A positive response was determined as follows. The mean of the ratio of the responses <2 was determined as well as the standard deviation from this mean. These individuals were considered nonresponders. Any response 2 SD > mean of the nonresponders was considered a positive response.

The duration of insulin administration was considered as having a potential effect on in vitro lymphocyte stimulation by the insulins. This factor was considered in relationship to the level of stimulation and to the category of response and nonresponse. No correlation was found.

HLA typing. HLA-A, B, C, and DR typing was performed on the peripheral blood lymphocytes from these patients by the microcytoxicity test described by Amos et al. (11). B lymphocytes for HLA-DR typing were isolated from the lymphocyte preparations using the Fab technique described by Mann et al. (12). Antisera that were used for detecting the HLA-DR determinants had previously been absorbed with pooled packed platelets or platelets with specific HLA-A, B, or C antigens. Assignments of the HLA determinants were based on the nomenclature of the seventh International Histocompatibility Workshop. The antisera used in this study defined 17 antigens controlled by the A locus, 30 antigens by the B locus, six HLA-C antigens, and eight antigens controlled by the DR locus. The frequency of each of the HLA tested for was determined in each group of individuals that demonstrated lymphocyte proliferation to antigen stimulation and was compared with the frequency of the same HLA antigen in the nonresponder group. Differences were examined for statistical significant by chi square analysis with Yates correction and applying the Fisher exact test (two-tailed) (13).

RESULTS

Insulin that is used for therapeutic purposes in the United States contains a variety of proteins that may serve as potential immunogens in man. Most insulin is derived from extractions of both beef and pork pancreases. While pork insulin differs in its amino acid composition from human insulin by only a single substitution (threonine for alanine) at the carboxy-terminal amino acid (B-30) of the B chain, beef insulin differs in three positions, the B-30 position and at positions 8 and 10 in the A chain (Table I). Therapeutic preparations also contain a small amount of beef and pork proinsulins. In these single chain precursors of the insulins, there is a 33–35 amino acid C-peptide between the A and B chains. These two C-peptides differ greatly in amino acid composition from each

TABLE I
Differences in Amino Acids in Three Mammalian Insulins

	A Chain (alpha loop)			B Chain
Insulin	Position 8	Position 9	Position 10	Position 30
Human	Threonine	Serine	Isoleucine	Threonine
Pork	Threonine	Serine	Isoleucine	Alanine
Beef	Alanine	Serine	Valine	Alanine

other as well as from that in human proinsulin. From animal studies, we know that each of the chains and the C-peptide represent domains of the molecule for which there is separate genetic recognition (6). Finally, many therapeutic preparations contain protamine, a basic protein extracted from fish sperm, which retards absorption of the insulins. The in vitro lymphocyte proliferation was performed using each of these five components and the results are shown in Figs. 1–6. Due to the overlapping sequence homologies of the various insulins and proinsulins, the data are presented to show the isolated response to each of the major therapeutic insulin components. Evaluation of the data in this manner is predicated on the observations made in guinea pigs (14). F1 hybrids of two strains, where one strain was responsive to beef and pork insulin (A and B chain response) and the other was responsive to pork insulin (B chain response), showed a greater lymphocyte proliferative response to beef insulin as compared with pork insulin. A similar increase in response was seen in the patients studied. This is illustrated in Fig. 1 where counts per minute of [^{3}H]thymidine incorporation is shown for those individuals who responded to beef insulin only and for those who responded to both pork and beef insulin but had a greater response to beef insulin than to pork insulin. Thus, those individuals that showed a response to beef insulin only and a greater response to beef than to pork were considered beef responders (A chain) and were grouped together for comparisons of HLA frequencies. The responses to each of the insulin components were converted to ratios and the results are presented in Figs. 2–6.

When comparing the individual patients, 48 patients showed a concordant response or nonresponse of their lymphocytes to antigenic stimulation by both beef and pork insulins (Fig. 2). This is true whether one considers the range of response, the mean of the response, or the individual response to these respective antigens. Since beef and pork insulins have only one difference from human insulin (the substitution of the B-30 amino

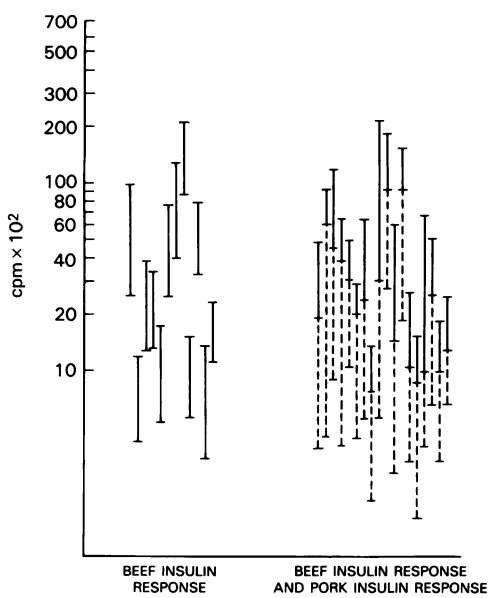

FIGURE 1 An example of lymphocyte response in counts per minute of individuals to beef insulin (| —— |) and pork insulin (| ----- |). In Figs. 2–6, cpm response to beef and pork insulins, proinsulins, and protomine are presented as ratios.

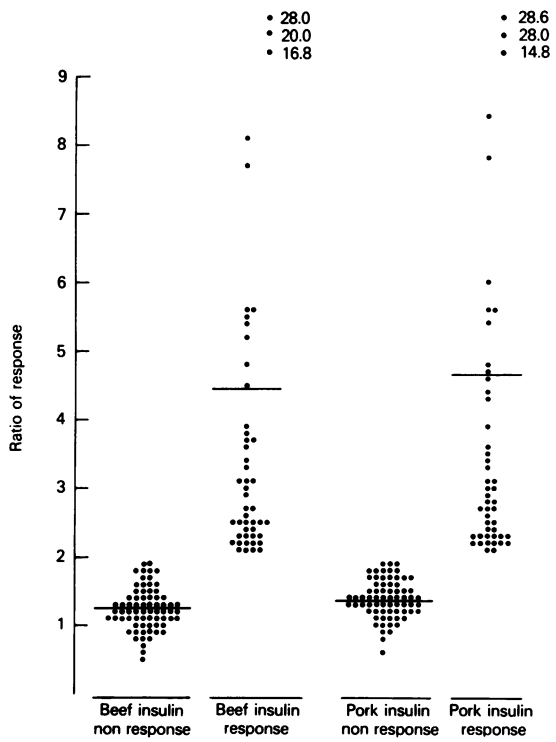

FIGURE 2 Ratios of lymphocyte proliferation response to beef and pork insulin stimulation. The mean ratio response and nonresponse is represented by the horizontal line. 48 individuals demonstrated near equal range and mean response to both insulins; this suggests recognition of the common carboxy-terminal amino acid shared by these insulins.

acid), these individuals appear to be responding to the B chain antigenic site.

For 30 individuals of this population, there was a selective recognition of beef insulin (Fig. 2). This was evident by a response to beef but not to pork insulin (12 patients) or by a response to beef insulin which was significantly greater than that to pork (18 patients). The ratio of response to beef was much greater than that to pork insulin (Figs. 1 and 3). The immune response of these individuals appears to be recognizing the A chain amino acid differences of the beef insulin molecule.

The C-peptide of proinsulin is known in animal studies to represent a separate domain of immune recognition, and this was reflected in the human proliferation studies as well. In 35 patients, a proliferative response was present for beef proinsulin but not for beef insulin, or the response was significantly greater to beef proinsulin than to beef insulin (Fig. 4); in 48 patients, a similar pattern was observed with pork proinsulin and pork insulin (Fig. 5). As with responses

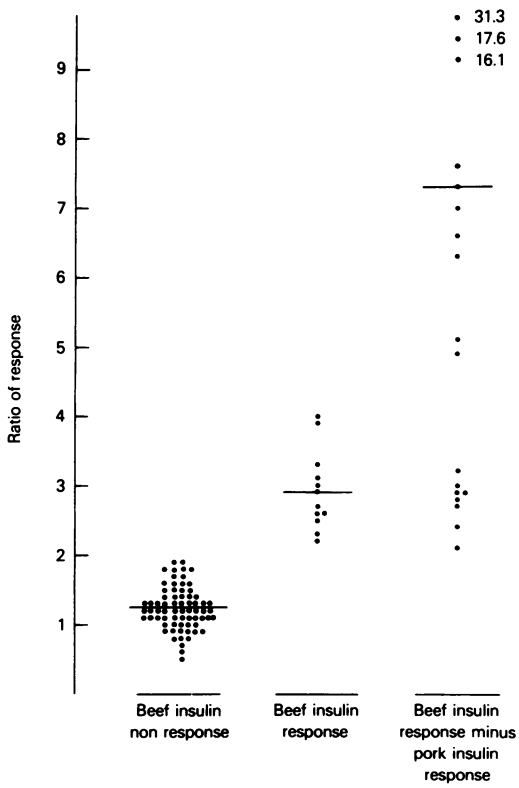

FIGURE 3 Ratios of lymphocyte proliferation response to beef insulins. Response was observed to beef insulin alone and in some individuals the ratio of response was greater to beef insulin than to pork insulin. The data suggest recognition of amino acid differences in the A chain of beef insulin.

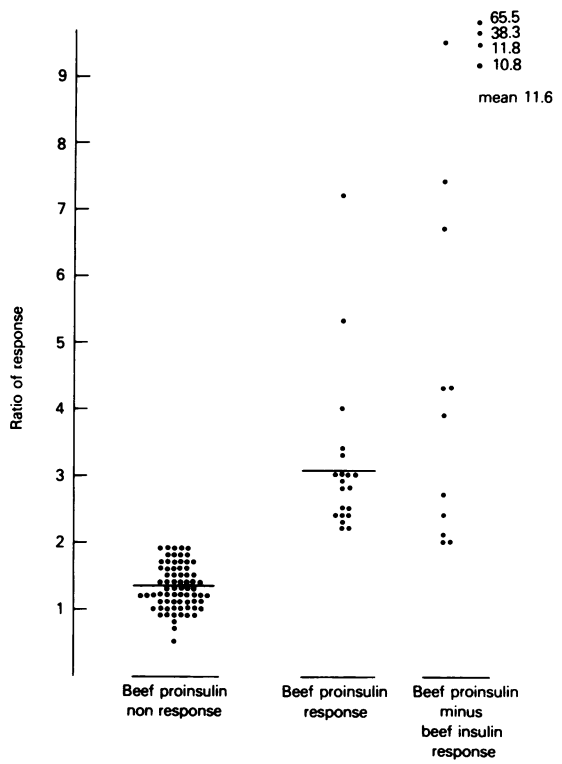

FIGURE 4 Ratios of lymphocyte response to beef proinsulin alone and an augmentation of the response to beef proinsulin over that seen with beef insulin alone.

to the individual insulins, there was a wide range in response ratios for the proinsulins.

Fig. 6 shows lymphocyte proliferation responses to protamine. Protamine is a histone protein, which is a constitutent of NPH and PZ-I insulins, that was added to delay the release of insulin once it has been administered. A restricted response to this protein was demonstrated in that there were individuals who appeared to respond and individuals who were nonresponsive in the in vitro lymphocyte proliferation studies.

The frequency of each of the HLA alloantigens was tabulated in each of the above groups. Those antigens which were found to be significantly different in frequency when comparing the responder to the nonresponder patients are listed in Table II. In this table, the frequency of the antigen is given in the two racial groups and in the total responder and nonresponder populations for each stimulating antigen. Each group of responders and nonresponders (Caucasian and Black) were analyzed for significant differences in HLA alloantigen frequencies. Only those differences that had P values ≤ 0.05 are shown.

Two antigens were altered in frequency in individ-

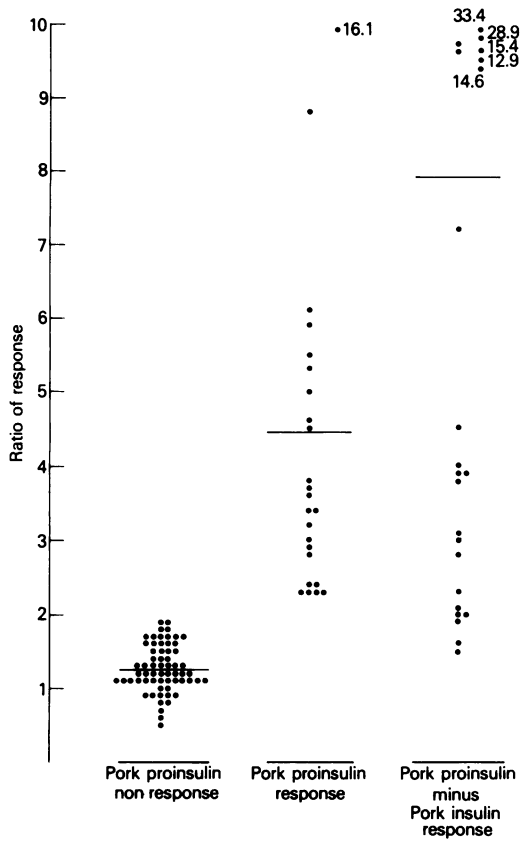

FIGURE 5 Ratios of lymphocyte response to pork proinsulin alone and incremental increases in response to the proinsulin above the response to pork insulin.

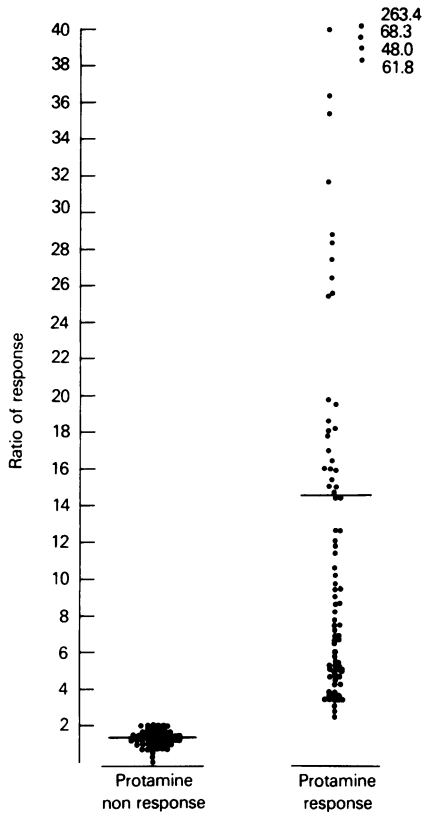

FIGURE 6 Ratios of lymphocyte response to protamine stimulation.

uals whose lymphocytes responded to beef insulin or had a greater response to beef than pork insulin. HLA-DR4 was significantly increased in frequency in beef insulin responders while HLA-DR5 was increased in frequency in the nonresponder populations. These data can be interpreted to demonstrate an Ir gene associated with the HLA-DR4 gene that recognized the amino acid differences in the A chain of beef insulin.

There were individuals whose lymphocyte response was such that there appeared to be HLA-associated recognition of the B chain differences in beef and pork vs. human insulin. This group included the 48 individuals whose lymphocytes responded equally to both insulins and 18 individuals who responded significantly to pork insulin, but whose response to beef insulin was greater than the pork response. In these responder groups, HLA-DR3 was increased in both Black and Caucasian populations. In comparing the frequency of this antigen in the total individuals that responded to beef and pork insulin, HLA-DR3 was found in 39% of the responder population and 17% in the nonresponder population. This difference was significant at the level of $P \leq 0.005$.

A positive proliferative response to the C-peptide domain of beef proinsulin was associated with several different HLA alloantigens. The most dramatic increase in antigen frequency was for HLA-CW5 which occurred in only 9% of nonresponders and 43% of responders. Significant increases were also observed in HLA-BW4, CW2, DR2, and DR5. The increases in CW2 and DR2 were most striking in the Black population, but the numbers were too small to be certain of the biological significance of the racial difference in response.

Although the C-peptide of beef and pork proinsulin differ from each other to almost the same extent that they differ from human C-peptide, the only HLA alloantigen positively associated with a pork proinsulin response was DR2, an antigen also associated with a beef proinsulin response. Again, this association was most dramatic in the Black population. In addition, two antigens, HLA-BW4 and DR3, were negatively associated with a pork proinsulin response.

HLA-A3 was decreased in frequency in the popu-

TABLE II

Comparison of HLA Alloantigen Frequencies in Responders and Nonresponders to Therapeutic Insulin Components

	Caucasian			Black			Total		
HLA antigen	Responder	Nonresponder	*P* value*	Responder	Nonresponder	*P* value	Responder	Nonresponder	*P* value
Beef insulin	*n* = 24	*n* = 83		*n* = 6	*n* = 23		*n* = 30	*n* = 106	
DR4	0.83	0.30	<0.001	0.33	0.17		0.73	0.27	<0.001
DR5	0.04	0.25	<0.05	0.17	0.26		0.06	0.25	<0.05
Beef and pork insulin	*n* = 52	*n* = 54		*n* = 14	*n* = 15		*n* = 66	*n* = 69	
DR3	0.42	0.16	≤0.005	0.36	0.20		0.39	0.17	≤0.005
Beef proinsulin	*n* = 27	*n* = 80		*n* = 8	*n* = 15		*n* = 35	*n* = 95	
BW4	0.88	0.56	<0.005	0.87	0.55		0.88	0.56	<0.002
CW2	0.18	0.10		0.50	0.00		0.25	0.08	<0.015
CW5	0.48	0.09	<0.001	0.25	0.10		0.43	0.09	<0.001
DR2	0.27	0.17		0.87	0.05	<0.025	0.40	0.15	<0.005
DR5	0.37	0.15		0.50	0.15		0.40	0.15	<0.005
Pork proinsulin	*n* = 31	*n* = 74		*n* = 12	*n* = 16		*n* = 43	*n* = 90	
BW4	0.52	0.70		0.50	0.81		0.51	0.72	<0.03
DR2	0.22	0.18		0.67	0.06	>0.008	0.35	0.15	<0.005
DR3	0.16	0.34		0.08	0.50		0.14	0.36	<0.01
Protamine	*n* = 63	*n* = 43		*n* = 15	*n* = 13		*n* = 78	*n* = 56	
A3	0.09	0.44	<0.001	0.25	0.31		0.13	0.41	<0.001
BW6	0.76	0.88		0.67	0.92		0.74	0.89	<0.001
CW3	0.38	0.14	<0.025	0.27	0.23		0.36	0.16	<0.025
CW5	0.27	0.07	<0.002	0.27	0.00		0.27	0.05	<0.005
DR7	0.32	0.12	<0.05	0.60	0.00		0.37	0.09	<0.001

* *P* values were calculated on all groups by methods described in the text. Only those values which are <0.05 are listed.

lations that responded to the antigen protamine. This difference was most obvious in the Caucasian population and was significant at the level of $P < 0.001$ when both populations were totaled. BW6 was also decreased in frequency in both the Black and Caucasian populations that responded to protamine. CW3 and CW5 were both increased in the Caucasian and Black populations that responded to this antigen. DR7 was found to be increased in frequency in both the Black and Caucasian responding populations. This antigen was found in 37% of 78 patients that responded to protamine and 9% of 56 nonresponders. The difference in frequency of this antigen in the two populations was significant at the level of $P \leq 0.001$.

DISCUSSION

The literature is replete with demonstrations of MHC-linked Ir genes in inbred strains of mice and guinea pigs. The majority of these observations are based on studies using synthetic polypeptide chains which tend to limit the antigenic determinants that can be recognized by the particular animal species. In addition, the use of congenic strains of mice tends to consolidate the potential Ir genes, and therefore eliminates the possible confusion that might take place due to gene interaction within the mice. An example of such an interaction has been reported by Berzofsky and coworkers in their studies of murine Ir genes to myoglobulin (15).

In contrast to the description of Ir genes in mice and guinea pigs, the demonstration that the human MHC controls immune response has been difficult. This is most probably due in part to the complexity of the MHC in our outbred species. This complexity generates a heterogeneity of potential control of the immune response which may have either helper and/or suppressor action. The problem is compounded by the multiplicity of determinants in the potential immunogens to which man is exposed. The intentional im-

munization of humans with antigens with restricted immunogenic determinants is generally not considered ethical. Thus, demonstration of Ir genes in man is relegated to exploration of situations where potential immunization exists through therapeutic efforts, such as insulin administration, vaccination, or substances that play an etiologic role in disease pathogenesis or manifestation (16–18).

Response to allogens that affect atopic allergic reactions have demonstrated an association with HLA. Mendell et al. reported an association of the HLA-A2 antigen with a positive skin test response to the RA3 antigen in individuals with low antigen-E response and a negative association with the same HLA antigen among individuals with an antigen-E response (19). The HLA-DW2 association with the RA5 ragweed allergens was also demonstrated by Marsh and colleagues (20). Griffing et al. demonstrated an association of HLA-DR3 with antibodies to DNA in patients with systemic lupus erythematosus (21). Hsu et al. presented evidence for potential genetic control of the immune response to synthetic polypeptides in man; this demonstrated that levels of lymphocyte proliferative response to these antigens are associated with particular HLA haplotypes (22). These studies suggest that the human Ir genes, in fact, map in the MHC; however, no association was demonstrated with a particular HLA alloantigenic determinant.

There are several reports that show an association of HLA alloantigens with antibody production to insulin in diabetic patients. Schernthaner and Mayr (23) as well as Bertrams and Grunklee (24) have studied patients with juvenile onset diabetes (JOD) and demonstrated that the development of antibodies to insulin was increased in frequency in individuals who were HLA-DR4 and decreased in frequency in individuals who were HLA-DR3 (10). These studies employed bovine insulin to measure insulin antibodies. Since lymphocyte proliferation in response to antigenic stimulation may represent the response of helper T cells, these studies complement our studies in that an increased HLA-DR4 frequency was observed in those individuals whose lymphocytes responded to beef insulin. When considered together, these studies suggest that the human immune system is capable of recognizing the two amino acid differences in beef and human insulin in the A chain of the insulin molecule and that there are Ir genes that are closely associated with the gene controlling HLA-DR expression. This argument is strengthened by similar observations in the above two groups selected on the basis of onset of diabetes, JOD, and the other group (this study) selected on basis of insulin administration which includes both JOD and mature onset diabetes.

We also observed a positive association of HLA-DR3 with the in vitro lymphocyte proliferative responses to beef and pork insulin. These insulins have in common the carboxy-terminal amino acid which differs from the amino acid at that position in human insulin. No other reports exist examining the immune response in man to this specific region of the molecule. Our results would suggest that there is an Ir gene associated with the HLA-DR3 antigen that recognized this single amino acid difference in insulins and invoked an in vitro immune response.

Contrasting the HLA antigen frequencies in the beef proinsulin and pork proinsulin responders with the frequencies of HLA antigens in the individuals who responded to beef and pork insulin adds further evidence for selective Ir genes to these therapeutic insulin components. DR5 was increased in frequency in the beef proinsulin responders and decreased in frequency in individuals whose lymphocytes responded to the beef insulin. HLA-DR3 was decreased in frequency in those individuals who responded to pork proinsulin, but was increased in frequency in individuals who responded to beef and pork proinsulins. These results also point out the complexity of establishing evidence for Ir genes in man. By its very nature, the proinsulin molecule possesses the beef or pork insulin molecule. Although some regions of the molecule are probably covered by the C-peptide in the three-dimensional folding of the molecule, there were individuals who responded to both the beef or pork insulin and to the respective proinsulins containing beef or pork insulin backbones, but where the response to the latter was greater than that to the former. For the purpose of analysis, these individuals have been included in the responder population to both beef and/or pork insulin and to the proinsulins of the respective species. The HLA frequencies that differentiate the insulin-proinsulin responders might be considerably more clear cut if the problem were not confounded by multiple determinants of the antigen (i.e., beef; beef proinsulin) and the complexity of the Ir genes in the outbred human.

A strong suggestion that the lymphoproliferative response to the histone protamine is under control of the MHC is a new observation. These responses were observed not only in some diabetics "immunized" with protamine-containing insulin, but also in a segment of the normal population. The same HLA association (CW5 and HLA-DR7) were observed in the nondiabetic protamine responders (unpublished observation). Whether or not this proliferative response represents a primary response in the "nonimmunized," nondiabetic individuals remains to be determined. Certainly, the possibility exists that protamine may serve as an adjuvant in the presentation of the insulin molecule, and in fact augment the immune response to the insulin in those individuals with Ir genes for protamine. This hypothesis would predict that admin-

istration of the synthetic human insulins with protamine will result in an immune response (possibly antibody) to the insulin in selected diabetic patients.

Positive responses to each of the antigens used in this study showed an association with one or another of the HLA-DR antigens. This is the association that one might expect if the human Ir genes map with that portion of the MHC that controls the expression of "Ia-like" antigens. Antigens controlled by loci other than DR were also elevated in frequency in the responder populations to beef proinsulin and protamine. This could be due to a multiple gene effect on response or to linkage disequilibrium of the antigens involved. This question could not be addressed in that the number of patients evaluated was too small to calculate haplotype frequencies. In addition, the patient population may have altered linkage groups because of their disease. The HLA-A3 frequency in the Caucasian and Black responder/nonresponder populations to protamine may be an example of linkage disequilibrium in different racial groups where it was observed that this antigen has significant association in one racial group (nonresponders) and no association in another.

In general, the frequency of an HLA antigen in one race in the population that responded to a particular insulin or protamine was reflected in a similar increase or decrease in the other race. These results tend to substantiate the hypothesis of an MHC-associated gene(s) that regulated or controlled the lymphoproliferative response to therapeutic insulins in that HLA antigen association was consistent in the two races where the frequencies of the antigen differ.

The strong association of HLA-DR4 with in vitro lymphocyte proliferative response to beef insulin and the previously reported association of this HLA antigen with antibody production to beef insulin has potential therapeutic implications. The clinician may elect to use insulins other than beef-derived insulin in diabetics who type for the HLA-DR4 antigen, and thus avoid the complications of insulin resistance (due to antibody to insulin) and potential adverse allergic reactions.

The results of this study substantiate an association of genes in the MHC with an in vitro parameter of the immune response. Since bovine and porcine insulins have relatively restricted differences from human insulin, a potential model for further dissection of Ir genes in man is established.

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