Plant Foods Hum Nutr (2010) 65:396–402
DOI 10.1007/s11130-010-0199-8

ORIGINAL PAPER

IgE-Mediated Cross-Reactivity among Leguminous Seed Proteins in Peanut Allergic Children

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Published online: 16 November 2010

Abstract The immunological cross-reactivity among major protein- and oil-crops, including lupin, lentil, pea, peanut, kidney bean and soybean, has been studied by a combination of *in vitro* and *in vivo* experimental approaches: SDS-PAGE separations of legume protein extracts and immuno-blot revelations with 12 peanut-sensitive subjects' sera, Immuno-CAP and Skin Prick tests on the same subjects. The immuno-blotting data showed a wide range of IgE-binding responses both displayed by one subject towards different plant extracts and among subjects. Differences were both quantitative and qualitative. The prevalent responses of most subjects' sera were seen with peanut polypeptides, as expected, as well as with various polypeptides of the other legumes, the most recurrent of which were the basic subunits of the 11S globulins. The distribution of *in vivo* responses generally paralleled those obtained by *in vitro* approaches with strong responses elicited by peanut, lentil and pea protein extracts, especially by most sensitive subjects, thus providing a consistent overall set of results. In this work, the comparison of various approaches has allowed us to get an overall broad picture of the immunological cross-reactivities among proteins of widely used different seed species and to hypothesize the role of most conserved specific polypeptides.

Keywords Antigenicity · Legume seeds · Food allergy · Cross-reactivity · *In vitro* test · *In vivo* test

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Introduction

Despite the consistent drop of leguminous seed production and consumption in many countries in the recent years [1], these crops represent a valuable and sustainable source of oil and proteins for food and feed. Moreover, the interest in various seed components, including oil, proteins, starches, fibres and other minor constituents, is currently expanding in parallel with the improvement of industrially-available separation techniques. Concurrently, the unveiling of biological activities of specific protein and non-protein components relevant to the consumers' health promotion is further enhancing the interest toward the legume seeds [2, 3].

On the other hand, the increased exploitation and diffusion of legume seeds as food ingredients and the inclusion of various cereals and legumes in the development of infant food supplements [4] require the careful evaluation of potentially adverse effects related to their consumption. In this respect, the analysis of the allergenic

potential of legume proteins is crucial. Among others, an important problem with potential clinical consequences is the immuno-cross-reactivities between legume seed proteins, which is based on similarities of their sequence and molecular structure. The common evolutionary origin of the 7S and 11S storage globulins in the cupin superfamily is one of the possible reasons of the observed cross-reactivity [5] and the molecular determinants responsible for their IgE-binding capacity have tentatively been identified [6]. As a matter of facts, cross-reactivities among some legume seeds, including soybean and peanut [7], soybean, lentil, pea and bean [8], lupin, pea and soybean [9], lupin and peanut [10], as well as with specific protein classes, such as pea and lentil vicilins and convicilins [11], pea and peanut vicilins [12], have been studied using various approaches. Some of these findings have recently been reviewed [13]. However, a systematic comparison of the most and/or increasingly diffused seed proteins with respect to their immunological and clinical cross-reactivities has yet to be accomplished.

In this work, we present the results of a study comparing *in vitro* IgE-binding and *in vivo* reactivities in a group of peanut-allergic children by using immunoblotting, Immuno-CAP and Skin Prick tests.

Materials and Methods

Legume Seeds

Legume seeds included in this studies were: lupin (*Lupinus albus*), lentil (*Lens culinaris*), pea (*Pisum sativum*), commercial roasted peanut (*Arachis hypogaea*), kidney bean (*Phaseolus vulgaris*) and soybean (*Glycine max*). The samples were finely ground to flour and prepared as described in the section related to the specific tests.

Patients

The design and aims of the study were authorized by the institutional review board of the Macedonio Melloni Hospital, Milan, Italy. Patients and their parents were provided with full information before they signed the consent form.

Twelve patients (11/1 male/female) admitted for immediate symptomatic onset after peanut exposure between 1 December 2006 and 30 June 2007 were considered, when they presented for follow-up visits. According to hospital internal protocols, children were diagnosed as allergic to peanut in case of positivity both to oral challenge and to Skin Prick test and/or Immuno-CAP to peanut proteins. However, all subjects showed reliable clinical reactions to peanut seeds, including angioedema, urticaria, asthma and gastrointestinal symptoms. Two of them experienced anaphylactic shock.

As negative controls, sera from milk-allergic and non-allergic children were used.

Skin Prick Test (SPT)

Skin Prick tests (SPT) were performed using freshly prepared allergens. To this purpose, legume flours were suspended in the SPT vehicle to obtain a 5% final protein concentration. The protein concentration of legume flours was experimentally determined by the Kjeldahl method [14, 15]. The vehicle contained 2% mannitol, 5.9% NaCl, 58% glycerol and 0.4% phenol and was sterilized by microfiltration.

Wheal diameters were measured by a caliper disk marked in millimetres 15 min after testing, and a test was considered positive when the wheal was at least 3 mm wide [16]. Histamine phosphate (ALK-Abellò, Copenhagen, Denmark), 10 mg/ml in 50% glycosaline solution and SPT vehicle alone were used as positive and negative controls, respectively.

Specific IgE Determination by Immuno-CAP®

Specific IgEs were measured in patients' sera from venous blood samples obtained at the time of enrolment. Levels of IgEs specific for peanut and other legumes included in the study were measured by an Immuno-CAP® test (Phadia, Upssala, Sweden). Allergen-specific IgE titres above 0.35 kU/l were considered as positive.

SDS-PAGE and Immunoblotting

The pattern of specific IgE binding to legume proteins was determined by immunoblotting using patients' sera.

Legume flours were suspended in sample buffer (containing 0.125 M Tris–HCl pH 6.8, 3.75% glycerol, 1% SDS and 5% β-mercaptoethanol) at a final protein concentration of 1.5 mg/ml. Legume proteins were then separated in 15% polyacrylamide gel according to Restani et al. [17], and transferred from the gel onto a polyvinylidene difluoride membrane (PVDF, Immobilon P, Millipore, Bedford, MA) by electrophoretic elution. The transfer buffer was 25 mM Tris–HCl, 193 mM glycine and 20% methanol. PVDF membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (Biorad, Italy) in 50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl, 5 mM EDTA and 0.05% Triton-X. The membranes were washed with 10 ml of 0.25% gelatin (Biorad) solution containing 0.3 ml of allergic patients' serum. Antigen-IgE complexes were detected using goat antihuman IgE antibodies labeled with alkaline phosphatase (Sigma Aldrich Italia).

After incubation in bromochloroindolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) solution, an intense precipitate developed at the site of the complex formation. All membranes were revealed with identical procedure and exposure time.

N-Terminal Amino Acid Sequencing

The electrophoretic gel was transferred to polyvinylidene difluoride membrane (PVDF, Immobilon P, Millipore, Bedford, MA) by blotting on a Trans-blot Electrophoretic Transfer Cell (Bio-Rad, Milan, Italy). The bands of interest were excised with a rasor blade. Automated sequence analysis was performed on a pulsed-liquid sequencer equipped with a PTH analyser (Procise model 491 Applied Biosystems, Foster City, CA). Similarities with the entries in the non-reduntant GenBank were searched using the BLAST program.

Results and Discussion

The SDS-PAGE patterns under reducing conditions of the protein extracts from the leguminous seeds considered in this work (lupin, lentil, pea, peanut, kidney bean and soybean) are shown in Fig. 1. The electrophoretic patterns of legume seed proteins are notoriously complex due to their multigene origin and hetereogeneity [18]. This heterogeneity and the incompleteness of genomic sequences, which is acute for some species among those considered in this work, often prevents mass and/or sequence analyses to be effective, as it occurs for many other procariotic and eucariotic organisms. Conversely, most legume seed proteins belong to well characterized protein families, as far as their electrophoretic behaviour and relative abundance is concerned. In particular, the legumin-like (11S) globulins consist of a few polypeptides which, under reducing conditions, separate into a lighter group (20–25 kDa) and a heavier group (35–50 kDa) [19]. The vicilin-like (7S) globulins are typically made up by a large number of polypeptides, covering a wide range of molecular masses which spanned from about 10 to 70 kDa, *i.e.,* lupin vicilin precursors of 55–65 kDa and products thereof of 12 kDa [20, 21], lentil vicilin of 47 kDa [22], pea vicilin of 50 kDa precursor and proteolysis products of 30, 20 and 12.5 kDa [23], peanut vicilin of 64 kDa [24], kidney bean phaseolins of 53, 47 and 43 kDa [25], and the three soybean α', α, and β-conglycinins of 71 kDa, 67 kDa and 50 kDa [26]. Together, the 11S and 7S globulins make up the large majority of seed proteins in most leguminous seeds and to many of them an allergenic potential have already been attributed, as summarized in Table 1.

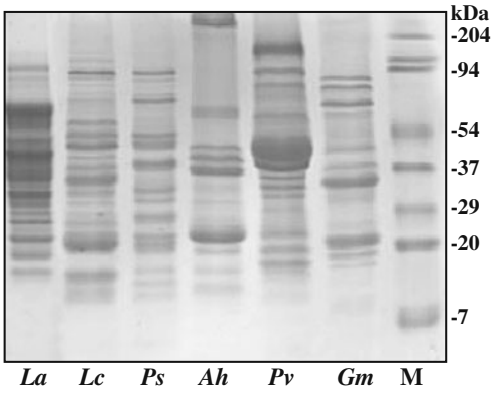

Fig. 1 SDS-PAGE of selected legume seed protein extracts. The panel shows the Coomassie blue stained gel of the protein extracts, run under reducing conditions. Sample lanes are the following: *La: Lupinus albus* (lupin); *Lc: Lens culinaris* (lentil); *Ps: Pisum sativum* (pea); *Ah: Arachis hypogaea* (peanut); *Pv: Phaseolus vulgaris* (kidney bean); *Gm: Glycine max* (soybean); M: Prestained marker proteins

While the electrophoretic profiles are reported in Fig. 1, the blotted membranes of the separated legume seed protein extracts reacted with the 12 peanut-sensitive patients' IgEs are compared in Fig. 2. The patterns of the immuno-blotted membranes reflect the complexity of the original Coomassie blue-stained gel and dramatically differ from one serum to another as the result of variable individual responses. Qualitative and quantitative differences in the reactivities were also visible within each serum, with the most prevalent responses of peanut extracts, being the subjects classified as peanut-sensitive. Nevertheless, especially in one case, *i.e.,* subject 102, the IgE-binding response of polypeptides belonging to two different species, namely lentil and pea, was even stronger. Although intensities of bands in membrane blotting analyses cannot be regarded as fully quantitative, the identical gel loading and revelation procedure adopted in this work, as mentioned under Methods, provided comparable pictures of relative IgE binding abilities. One of the most responsive and recurrently detected peanut polypeptide in all patients was a 20 kDa polypeptide. In order to unequivocally identify this polypeptide, N-terminal amino acid sequence analysis of the excised band was performed, as described under Methods. The sequence obtained, *i.e.,* GIEETIX clearly identifies the polypeptide as the basic subunit of arachin, the peanut legumin-like globulin (Accession Ns. Q647H2, Q5I6T2, Q6T2T4, B5TYU1, A1DZF0, Q647H4, Q647H3 at www.expasy.ch). A certain IgE-binding capacity was also seen with a 37 kDa peanut polypeptide in most subjects. For this reason N-terminal amino acid sequence analysis was extended to this band. The sequence obtained, *i.e.,* ISFRQQPEEN, allowed the attribution of this polypeptide to the acidic subunit of arachin (Accession Ns. Q5I6T2, Q6T2T4, B5TYU1, A1DZF0, Q647H4, Q647H3

Table 1 Major legume seed globulin families and identified allergenic polypeptides

Seed source	Globulin family (Common name)	Allergenic polypeptide	
		Name	M_r, kDa
Lupin (*Lupinus albus*)	7S (β-conglutin)	Lup an 1[a]	55–61[a]
		Lup a vicilin[b]	n.a.
		Lup a β-conglutin[b]	n.a.
	11S (α-conglutin)	Lup a11S globulin[b]	n.a.
Lentil (*Lens culinaris*)	7S (vicilin-like)	Len c 1[a]	47[a]
	11S (legumin-like)	n.a.	n.a.
Pea (*Pisum sativum*)	7S (vicilin)	Pis s 1[a]	44[a]
	11S (legumin)	Pis s 2[a]	63[a]
Peanut (*Arachis hypogaea*)	7S (conarachin)	Ara h 1[a]	64[a]
	11S (arachin)	Ara h 3[a]	60[a]
		Ara h 4[a]	37[a]
Kidney bean (*Phaseolus vulgaris*)	7S (phaseolin)	n.a.	n.a.
	11S (legumin-like)	n.a.	n.a.
Soybean (*Glycine max*)	7S (β-conglycinin)	Gly m 5[a] (β subunits)	48
		Gly m 5[a] (α subunits)	63
		Gly m 5[a] (α′ subunits)	65
	11S (glycinin)	Gly m 6 G1subunit[a]	54
		Gly m 6 G2 subunit[a]	52
		Gly m 6 G3 subunit[a]	52
		Gly m 6 G4 subunit[a]	61
		Gly m 6 G5 subunit[a]	56

n.a. not assigned

[a] available on the Web at http://www.allergen.org

[b] available on the Web at http://www.allergome.org

at www.expasy.ch). These findings are in agreement with reference data of Table 1 and with previous results showing that the major (15 patients) or unique (5 patients) peanut allergen for 16 investigated Italian children was this Ara h 3 polypeptide constituent [27]. In that work, the identification of the reactive Ara h 3 basic subunits from other peanut low M_r polypeptides, consisting of Ara h 2 allergens, was achieved thanks to the great resolution power of 2D electrophoresis. Apparently, strong reactivity of the legumin-like basic subunits occurred also with most other species, especially lentil, pea, and soybean, where the IgE-binding to legumin basic subunits was clearly visible and shared among practically all subjects' sera. A similar *in vitro* IgE binding ability had already been observed in lupin sensitive-subjects with soybean, peanut and pea [28]. Recent findings have confirmed the strong reactivity of lupin legumin with the IgEs of peanut-allergic patients; however, in that case a greater response of the large acidic legumin subunits rather than the basic one [29], as predominantly seen in the present work, was detected.

As far as IgE binding ability to vicilin polypeptides is concerned, the identification of specific bands was hampered by the already mentioned extreme heterogeneity of this protein family. Generally speaking, due to the absence of disulphide bonds in the proteins of this group, the largest most abundant polypeptides in the membranes seemingly corresponded to the vicilin-like intact polypeptides. With lentil and kidney bean samples, attribution was facilitated by the unique presence of vicilin proteins, as main storage protein, in these seeds [22, 25]. In these cases, however, the overall intensities were generally lower. Also α′, α, and β soybean conglycinins did not show strong reactivities.

Another interesting finding concerned the almost absent detection of responsive bands below 20 kDa in most membranes, with only few exceptions (subjects 102, 103 and 105). Under the denaturing and reducing conditions used in this work, most of the 2S seed proteins have M_r slightly lower than this value, as shown in ref. [27]. However, other authors have detected unprocessed precursor of the 2S proteins around 20 kDa strongly reacting with their patients' population [30]. The present findings support

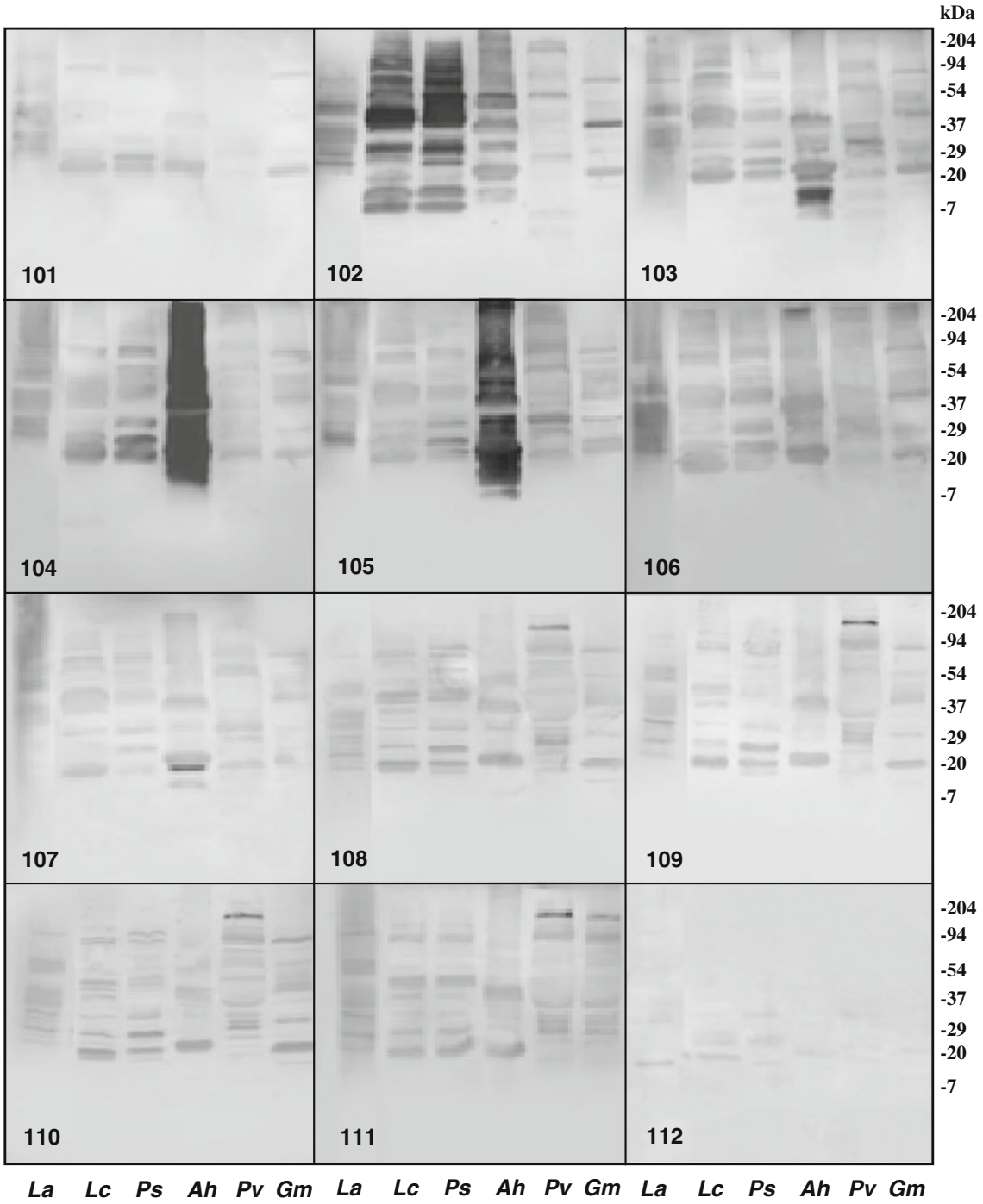

Fig. 2 Immunoblotting analysis of legume seed protein extracts. Panels show the membranes reacted with peanut-sensitized patients circulating IgEs. Experimental details are given in Material and Methods. *Numbers* indicate each patient. Sample lanes are the following: *La: Lupinus albus*; *Lc: Lens culinaris*; *Ps: Pisum sativum*; *Ah: Arachis hypogaea*; *Pv: Phaseolus vulgaris*; *Gm: Glycine max*

the conclusion that, at least in our patients' cohort, the 2S seed proteins are not major allergens. This lack of immuno-response by the 2S proteins has already been noted in lupin, soybean and peanut [28].

No positive responses were observed with sera from milk-allergic or non-allergic children subjects (not shown).

The results shown in Fig. 2 are consistent with the trials on patients performed using the protein extracts of each legume seed considered in this work. Indeed, Table 2 shows the results obtained with the 12 patients by using legume seed protein extracts in Skin Prick test and Immuno-CAP tests. A close qualitative correlation between the intensities

Table 2 Skin Prick (SP) and Immuno-CAP (IC) trials with legume seed extracts on 12 peanut-sensitive subjects

Subject	La		Lc		Ps		Ah		Pv		Gm	
	SP	IC	SP	IC	SP	IC	SP	IC	SP	IC	SP	IC
101	n	0	n	0	3	0	4	0	3	0	n	0
102	7	40.8	6	>100	6	>100	6	20.6	4	13.0	6	35.7
103	n	8.9	3	11.0	n	1.8	n	27.9	3	12.2	3	6.0
104	n	1.0	n	3.3	n	1.6	10	99.7	n	1.8	n	2.9
105	n	18.3	3	18.4	5	9.9	6	>100	4	19.7	4	19.6
106	3	0	3	0	6	0	7	1.1	3	0	6	0
107	5	3.1	5	5.8	4	3.9	3	7.9	5	2.5	5	6.8
108	n	0	n	0	n	0	13	0.4	3	0	n	0
109	3	1.5	15	1.6	10	0.6	3	5.4	4	1.6	6	1.2
110	4	3.4	6	3.7	6	1.2	7	5.5	8	0	3	2.3
111	5	2.7	7	3.5	7	1.7	5	5.0	6	0.8	7	2.6
112	5	0	3	0	n	0	3	0	n	0	3	0

La *Lupinus albus*; Lc *Lens culinaris*; Ps *Pisum sativum*; Ah *Arachis hypogea*; Pv *Phaseolus vulgaris*; Gm *Glycine max*

Prick test: results were expressed in mm. Values <3 mm were considered as negative (n).

Immuno-CAP test: results were expressed as kU/L. Values <0.35 kU/L were considered as negative.

of the bands in the membranes of Fig. 2 and Prick and Immuno-CAP responses in each patient can be drawn. These correlations are schematically listed here below:

- Subject 102 had strong responses specially with lentil, pea, lupin and less with peanut, kidney bean and soybean both *in vitro* and *in vivo*;
- Subjects 103, 104 and 105 showed low response with all legume seeds, but very strong reactions almost exclusively with peanut both *in vitro* and *in vivo*;
- Subject 106 showed no apparent clinical reaction despite relatively intense membrane staining;
- Subject 101 and all other subjects, *i.e.,* from 107 to 112, had remarkably lower reactions with most extracts both in the immunoblottings and in the biological assays.

Therefore, the two different experimental approaches used appeared mutually supportive.

In the case of lupin proteins, previous results obtained by comparing immuno-blot, Skin Prick and Immuno-CAP tests of peanut-sensitive patients were also remarkably consistent, with only two patients out of 12 showing clinical responses in the oral challenge [31].

Conclusions

This work presents the results of *in vitro* and *in vivo* experimental work aimed at unveiling the immunological cross-reactivity of peanut-sensitive subjects with other widely or increasingly consumed legume seeds. In a previous study, it was shown that peanut-sensitized adult patients frequently showed clinically relevant sensitization to either lupin, pea or soybean [32], however no attempt to identify specifically active polypeptide constituents was presented. The results of *in vitro* and *in vivo* approaches presented here are mutually consistent and show a common pattern of reactivities, specially related to polypeptides from the legumin globulin family, as the result of their interspecies sequence and structure similarities. The emerging overall broad picture highlights remarkable cross-reactivities of the legume seeds considered in this work and focus the great immunoreactivity of the legumin-like basic subunit, which has already been noted for prevalent and recurrent responses in our population.

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